AN INNOVATIVE INTEGRATED APPROACH TO CHARACTERIZE COFFEE RESISTANCE MECHANISMS TO *COLLETOTRICHUM KAHAWAE*

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Abstract

Coffee berry disease, caused by the hemibiotrophic fungus *Colletotrichum kahawae*, is a major constraint to Arabica coffee production in Africa. Coffee variety Catimor 88, which exhibit field resistance in Kenya, was selected to characterize the resistance to *C. kahawae*, comparatively to the susceptible variety Caturra. Hypocotyls of both varieties were challenged with *C. kahawae* (isolate Que2 from Kenya) and samples were collected during infection time-course, simultaneously for analysis of fungal growth and plant responses (light microscopy), evaluation of enzymatic activities (spectrophotometry, electrophoresis, histochemistry) and gene expression analysis (quantitative real-time PCR).

The resistance was characterized by restricted fungal growth associated with the hypersensitive reaction and early accumulation of phenolic-like compounds in the cell walls and cytoplasmic contents. Similar responses were detected in the susceptible variety but in a significantly lower percentage of infection sites. Regarding the genes related to the salicylic acid, jasmonic acid (JA) and ethylene (ET) pathways (phytohormones biosynthesis, reception, and responsive-related genes), this study suggests the involvement of JA in the resistance while ET seems to be more related with the susceptibility.

The expression of genes related to recognition and signaling (*RLK*, *LRR-K*, *CML*, *PTL*) and cell wall modification genes (*PME41, MUR4*) was induced in both coffee varieties, at early stages of the infection. However, in the resistant variety, a higher expression of recognition and signaling genes was induced together with the *PME41* gene during fungal penetration, and the induction of expression of the Lignin-forming anionic peroxidase-like gene (*PER4*) was supported by the increase of total peroxidase activity and of an anionic isoform. Peroxidase was localized in the walls and cytoplasmic contents of host cells, at the infection sites. The new data obtained enable to identify potential biomarkers of disease resistance that, once validated, will be useful for marker-assisted selection in coffee breeding programmes.

Key-words: Coffee Berry Disease (CBD), *Coffea* sp., cytology, biochemistry, gene expression

Resumo

A antracnose dos frutos verdes, causada pelo fungo hemibiotrófico *Colletotrichum kahawae*, é a principal doença do cafeeiro Arábica, em África. A variedade Catimor 88, que apresenta resistência de campo no Quénia, foi selecionada para caracterizar a sua resistência a *C. kahawae,* em comparação com a variedade suscetível Caturra. Hipocótilos das duas variedades foram inoculados com *C. kahawae* (isolado Que2, Quénia) e colheram-se amostras simultaneamente para avaliação do crescimento do fungo e respostas da planta (microscopia óptica de campo claro e de fluorescência), determinação de atividades enzimáticas (espectrofotometria, eletroforese e histoquímica) e para análise de expressão de genes (PCR em tempo-real).

A resistência caracterizou-se pelo crescimento restrito do fungo associado à reação de hipersensibilidade e à acumulação precoce de compostos fenólicos nas paredes celulares e nos conteúdos citoplasmáticos. Estas respostas foram observadas na variedade suscetível, mas numa percentagem significativamente inferior de locais de infeção. A expressão de genes relacionados com as vias do ácido salicílico, ácido jasmónico (JA) e etileno (ET) (biossíntese, recetores e genes induzidos pela fitohormona) sugere o envolvimento do JA na resistência enquanto o ET parece estar mais envolvido na suscetibilidade.

Os genes de reconhecimento, sinalização (*RLK*, *LRR-K*, *CML*, *PTL*) e genes envolvidos em modificações nas paredes celulares (*PME41, MUR4*), foram induzidos em ambas as variedades, no início do processo de infeção. Na variedade resistente, durante a penetração do fungo observou-se um aumento da expressão dos genes referidos, excepto para o gene *MUR4.* Observou-se ainda a indução do gene *Lignin-forming anionic peroxidase-like* (*PER4*) suportada pelo aumento de actividade total da peroxidase e de uma isoforma aniónica. A peroxidase foi localizada nas paredes e no conteúdo citoplasmático das células do hospedeiro, nos locais de infeção.

Os dados obtidos permitiram identificar potenciais biomarcadores de resistência que, após validação, serão úteis para a seleção assistida por marcadores em programas de melhoramento genético.

Palavras-chave: antracnose dos frutos verdes, *Coffea* sp., citologia, bioquímica, expressão génica

Resumo alargado

O café é um dos produtos agrícolas com maior valor comercial, representando uma importante fonte de rendimento para muitos países. A sua produção, processamento e comercialização emprega centenas de milhões de pessoas em todo o mundo (ICO-International Coffee Organization, www.ico.org).

A antracnose dos frutos verdes do cafeeiro, vulgarmente conhecida por Coffee Berry Disease (CBD), é causada pelo fungo hemibiotrófico *Colletotrichum kahawae* J.M. Waller& P.D. Bridge e constitui o principal fator limitante do cultivo de cafeeiro Arábica (*Coffea arabica* L.) em África. Os frutos infetados apresentam necroses em depressão com esporulação e podem cair prematuramente ou permanecer mumificados nos ramos do cafeeiro. Esta doença pode causar 50-80% de perdas de produção e o seu controlo tem sido conseguido essencialmente por meio de luta química, que tem um efeito reconhecidamente nefasto no ambiente, para além de ser bastante dispendioso. O cultivo de variedades resistentes é considerado uma forma alternativa e sustentável de combater esta doença. Os primeiros programas de melhoramento com vista à obtenção da resistência ao CBD iniciaram-se há cerca de 35-40 anos no Quénia, Etiópia e Tanzânia. Em Portugal, no Centro de Investigação das Ferrugens do Cafeeiro – CIFC (unidade de investigação do ISA, Universidade de Lisboa), desde 1989 que se têm desenvolvido estudos sobre a resistência ao CBD, em estreita cooperação com os países produtores de café, destinada a apoiar os seus programas de melhoramento. De forma a selecionar plantas resistentes têm sido efetuadas, no CIFC, testagens em larga escala com isolados de *C. kahawae* de diferentes países Africanos. Estas testagens permitiram a identificação de progénies de cafeeiro simultaneamente resistentes ao CBD e à ferrugem alaranjada, nomeadamente em derivados do Híbrido de Timor (híbrido natural entre *C. arabica* e *C. canephora*).

As graves repercussões sócio-económicas do CBD em África e o risco da sua introdução em países produtores de café da América e Ásia, justificam a necessidade de se concentrarem esforços na pesquisa e caracterização de fontes de resistência de forma a que essa informação seja usada em programas de melhoramento genético.

O presente trabalho teve como objetivo caracterizar os mecanismos celulares, bioquímicos e moleculares de resistência da variedade Catimor 88 (derivado de Híbrido de Timor), que apresenta resistência a *C. kahawae* em condições de campo no Quénia. Foi usada comparativamente a variedade suscetível Caturra. Utilizaram-se hipocótilos, porque estudos anteriores mostraram existir uma correlação entre os testes em hipocótilos e a resistência dos frutos verdes no campo. Os hipocótilos de ambas as variedades foram inoculados com uma

suspensão de conídios de *C. kahawae* (isolado Que2, originário do Quénia) e foram colhidas amostras ao longo do processo de infeção, para avaliação simultânea do crescimento do fungo e respostas da planta (por microscopia ótica de campo claro e de fluorescência), determinação de atividades enzimáticas (por espetrofotometria, eletroforese e histoquímica) e para análise de expressão génica (por PCR em tempo-real).

Os estudos de microscopia mostraram não existir diferenças significativas nas percentagens de germinação de conídios e de formação de apressórios entre a variedade resistente e a suscetível. Em ambas as variedades, a penetração das células da epiderme ocorreu cerca das 48 horas após a inoculação (hai), a partir de apressórios melanizados com formação da vesícula de infeção e posteriormente de hifas intra- e intercelulares. Na variedade suscetível o fungo estabeleceu inicialmente uma interação biotrófica com as células da epiderme e do córtex que colonizou, tendo iniciado cerca das 72 hai um crescimento necrotrófico. O fungo prosseguiu o seu crescimento colonizando novas células do hospedeiro num ciclo de biotrofia/necrotrofia que culminou com o aparecimento dos sintomas. Pelo contrário, a resistência foi caracterizada por um crescimento restrito do fungo (as hifas ficaram confinadas nas células da epiderme ou ocasionalmente na primeira camada de células do córtex), como foi confirmado pela medição do crescimento médio das hifas/local de infeção. As primeiras respostas induzidas pelo fungo foram observadas, a partir das 24hai, em células da epiderme e corresponderam à reação de hipersensibilidade e à acumulação de compostos fenólicos nas paredes ou nestas e nos conteúdos citoplasmáticos. Estas respostas estenderam-se ao longo do tempo a outras células da epiderme e do córtex e foram também observadas na variedade suscetível, mas numa percentagem significativamente inferior de locais de infeção, quando comparada com a variedade resistente.

Tendo por base os resultados de microscopia, foram escolhidos tempos-chave do processo de infeção para os estudos de expressão de genes e determinação de atividades enzimáticas.

A análise da expressão de genes relacionados com as vias do ácido salicílico (SA), ácido jasmónico (JA) e etileno (ET) (biossíntese, recetores e genes induzidos pela presença da fitohormona) sugere o maior envolvimento do JA e do ET do que do SA na interação cafeeiro-*C. kahawae*. Com efeito, na variedade resistente, comparativamente com a variedade suscetível, verificou-se uma indução maior e em etapas mais precoces do processo de infeção dos genes *Oxophytodienoate redutase 3* - *OPR3* (biossíntese do JA), *Coronatine insensitive 1* - *COI* (recetor do JA) bem como *Pathogenesis-related genes* (*PR1*e *PR10*), o que sugere o envolvimento de JA na resistência.

Relativamente à via do ET, na variedade resistente a baixa ou não regulação dos genes *Ethylene resistant 1 - ETR1* e *Ethylene insensitive 2 - EIN2* (recetores do ET) e a moderada expressão do gene *Ethylene-responsive factor 1 - ERF1* (induzido pela presença do ET) parece indicar que esta fitohormona possa estar relacionada com outras funções para além da resistência. Todavia, na variedade suscetível, a maior ativação do gene *ERF1* no início da fase necrotrófica, coordenada pela ativação dos genes *1-aminocyclepropane-1-carboxylic acid synthase 5 - ACS5* e *1-aminocyclepropane-1-carboxylic acid oxidase 2 - ACO2* (biossíntese) e ainda pelos genes *ETR1* e *EIN2* (recetores), parece indicar que o ET seja mais relevante na expressão de suscetibilidade.

Foram ainda estudados os perfis de expressão de genes relacionados com o reconhecimento do agente patogénico e sinalização (*Receptor-like kinase – RLK, Leucine rich repeat receptor-like serine/threonine-protein kinase At2g16250 – LRR-K, Proline-rich receptor-like protein kinase – PERK3, Calmodulin-like protein – CML, Patatin-like phospholipase -PTL*), modificações da parede celular (*UDP-arabinose 4-epimerase 1-MUR4*, *Pectinesterase/pectinesterase inhibitor 41- PME41*) e genes da peroxidase relacionados com a produção de formas reativas de oxigénio (*Cationic peroxidase 2 – PNC2*) e com a lenhificação (*Lignin-forming anionic peroxidase-like – PER4*). Em ambas as variedades foi observada a indução dos genes relacionados com o reconhecimento do agente patogénico e sinalização (*RLK, LRR-K, PERK3, CML, PTL*) e com modificações na parede celular (*MUR4* e *PME41*) em fases precoces do processo de infeção (germinação de conídios e diferenciação de apressórios), provavelmente devido a uma resposta de defesa basal não específica. No entanto, durante a fase de penetração do fungo na variedade resistente, os referidos genes de reconhecimento e sinalização e o gene *PME41* relacionado com modificações na parede celular, à semelhança do que se verificou com os genes *COI, PR1* e *PR10* da via do JA, apresentaram um aumento significativo de expressão.

Em relação aos genes da peroxidase (*PNC2*, *PER4*) a sua expressão foi, em geral, regulada em todos os pontos do processo de infeção em ambas as variedades. No entanto, os valores de expressão de *PER4* foram maiores na variedade resistente do que na suscetível com diferenças significativas às 12hpi e aumentaram ao longo do processo de infeção. Estes resultados foram ainda suportados pelo aumento da atividade total da peroxidase e pela maior intensidade de coloração de uma isoforma aniónica (pI 6.1) apenas observada na variedade resistente. A peroxidase foi localizada nas paredes e no conteúdo citoplasmático das células do hospedeiro, nos locais de infeção. Foi igualmente detetado o aumento de atividade total da polifenol oxidase na variedade resistente, mas numa fase avançada do processo de infeção (72hpi), podendo eventualmente contribuir para as respostas de defesa mais tardias.

Neste estudo foram obtidos novos resultados particularmente sobre genes e proteínas envolvidos na expressão de resistência do cafeeiro a *C. kahawae*, que poderão ser validados como potenciais biomarcadores de resistência através de estudos funcionais. Uma vez validados, estes biomarcadores deverão ser testados noutros genótipos de cafeeiro com largo espectro de resistência a diferentes isolados de *C. kahawae*, de forma a avaliar o seu potencial como ferramenta auxiliar em programas de melhoramento.

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Abreviations

ACO	1-aminocyclepropane-1-carboxylic acid oxidase
ACS	1-aminocyclepropane-1-carboxylic acid synthase
AF	Autofluorescence
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
bp	Base pairs
BSA	Bovine serum albumin
BTH	Benzothiadizole
CBD	Coffee Berry Disease
cDNA	Complementary deoxyribonucleic acid
CML	Calmodulin-like protein
COI1	Coronatine insensitive 1
CTR1	Constitutive triple response 1
DDE2	Delayed dehiscence 2
DNA	Deoxyribonucleic acid
EIN2	Ethylene insensitive 2
ERF1	Ethylene-responsive factor 1
ET	Ethylene
ETI	Effector-triggered immunity
ETR1	Ethylene resistant 1
hai	Horas após inoculação
hpi	Hours post inoculation
HR	Hypersensitive reaction
ICO	International Coffee Organization
ICS	Isochorismate synthase
IDE	Insuline Degrading Enzyme
IEF	Isoelectric focusing electrophoresis
Ile	L-isoleucine
ISA	Instituto Superior de Agronomia
JA	Jasmonic Acid
JAR1	Jasmonate-resistant 1
JAZ	Jasmonate zim domain proteins
LRR-K	Leucine rich repeat receptor-like serine/threonine-protein kinase At2g16250
LRR-RLKs	Leucine-rich repeat receptor-like kinases
MEA	Malt extract agar
MeJA	Methyl jasmonate
MUR4	UDP-arabinose 4-epimerase 1
NPR1	Non-expressor of pathogenesis-related genes 1
OGs	Oligogalacturonides
OPDA	12-oxophytodienoic acid
OPR3	Oxophytodienoate redutase 3
PAD4	Phytoalexin-deficient 4

PAGE	Polyacrylamide gel electrophoresis
PAL	Phenylalanine ammonia-lyase
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase Chain Reaction
PER4	Lignin-forming anionic peroxidase-like
PERK	Proline-rich receptor-like protein kinase
PME41	Pectinesterase/pectinesterase inhibitor 41
PNC2	Cationic peroxidase 2
POD	Peroxidase
PPO	Polyphenol oxidase
PR	Pathogenesis-related protein
PRR	Pattern recognition receptor
PTI	PAMP-triggered immunity
PTL	Patatin-like phospholipase
qPCR	Quantitative polymerase chain reaction
RLK	Receptor-like kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S24	Ribosomal protein S24
SA	Salicylic Acid
SAM	S-adenosyl-L-methionine
SID2	Salicylic acid induction deficient 2
Ta	Annealing temperature
Tm	Melting temperature
α-LA	α-linolenic acid
β-Tub9	Tubulin beta-9

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Chapter 1

General introduction

1. General introduction

1.1. Coffee crop, its economic importance and geographic distribution

Coffee, the most important agricultural commodity, is crucial for the economy of many countries, which are largely dependent upon this commodity for their export earnings and for the achievement of their social and economic development goals (ICO − International Coffee Organization, www.ico.org/sustaindev_e.asp).

The genus *Coffea* belongs to the *Rubiaceae* family and currently includes 124 species native to Africa, India, Papua New Guinea, and Australasia (Davis *et al.,* 2011). The two main cultivated coffee species, *Coffea arabica* (Arabica coffee) and *C. canephora* (Robusta coffee), account for 63 and 37% of the world coffee production in average (www.ico.org).

Coffee is grown in the tropical and sub-tropical regions with a prevalence of *C. arabica* in South America, Central and Eastern Africa, India and some regions of Indonesia and *C. canephora* is widely produced in the low lands of many west African countries where it is native, but also in Southwest of Asia and in some regions of Brazil (www.ico.org). Arabica is well adapted to high altitudes (1000-2500 m), with raining seasons (1500-2000 mm annually) followed by dried seasons and temperatures between 15-24ºC (optimal temperature of 20ºC). Robusta is well adapted to low altitudes (0-700 m), heavy precipitation (2000- 3000 mm annually) and temperatures between 24 to 30ºC (optimal temperature of 27ºC).

Brazil, followed by Vietnam, Ethiopia and Honduras were the main coffee growing countries, producing up to 57% of the 152 million 60kg bags (over 9 million tons) in 2016. Coffee is mostly consumed in Europe, followed by Asia and Oceania and North America (www.ico.org). Arabica coffees are generally sold at twice the price of Robustas or even more, on account of superior beverage quality. However, costs of production are much higher, mainly due to more stringent demands for soil and climatic conditions, crop management, primary processing and control of several pests and diseases, including the potentially very destructive coffee leaf rust (or orange rust) and coffee berry disease, caused by the fungi *Hemileia vastatrix* Berkeley and Broome and *Colletotrichum kahawae* Bridge and Waller, respectively (Wintgens, 2009; van der Vossen *et al.*, 2015)

1.2. Coffee Berry disease

Coffee berry disease (CBD), caused by the fungus *C. kahawae*, is a major constraint of Arabica coffee production in Africa, where it is endemic (Hindorf & Omondi, 2011; van der Vossen *et al.*, 2015; Alemu *et al.*, 2016). Occurrence of CBD depends mostly on climate conditions and altitude ranges; regions of higher altitude are more prone to the disease than lower altitude regions, and mild temperatures favor the disease (Gichuru *et al.* 2008; van der Vossen & Walyaro, 2009). Identified for the first time in Kenya in 1922 (McDonald, 1926), CBD progressively spread throughout all the African coffee growing countries where is still restricted, turning the risk of its introduction in America and Asia in a major concern (Silva *et al.*, 2006; van der Vossen & Walyaro, 2009).

C. kahawae (epithet derived from the Swahili word for coffee), is very closely related to other members of the *C. gloeosporioides* species complex, but only *C. kahawae* is capable of infecting green coffee berries (Firman & Waller, 1977; Beynon *et al.*, 1995; Pires *et al.*, 2016; Batista *et al.*, 2017). Other *Colletotrichum* species (including other members of the *C. gloeosporioides* species complex along with *C. acutatum*) are also found in coffee berries, but they act as saprobes by invading mature coffee berries and are unable to infect green berries (Chen *et al.*, 2003). In fact, a phylogenetic analysis has shown evidence of a recent host jump underlying the speciation of *C. kahawae* from a population of *C. gloeosporioides sensu lato* isolates from diverse hosts (Silva *et al.*, 2012).

C. kahawae is able to infect several coffee organs, from flowers to ripe fruits and leaves, but major losses result from the infection of green developing berries leading to the formation of dark sunken lesions with sporulation (acervuli) (Fig. 1.1) , followed by their premature dropping and mummification (Silva *et al*., 2006, Hindorf and Omondi, 2011).

Figure 1.1 - A susceptible coffee variety showing disease symptoms

This disease may cause up to 50-80% of crop losses, in years of prolonged wet and cool weather conditions if no preventing control is applied (McDonald, 1926; van der Vossen *et al.*, 1976; Silva *et al.*, 2006; van der Vossen & Walyaro, 2009). For such huge economic impact, it is ranked as a quarantine pathogen and even as a biological weapon (Australia Group, 2014; Batista *et al.*, 2017).

Chemical control is the most common way of disease management. However, fungicide application can lead to environmental pollution and emergence of fungicide-tolerant strains, as well as it can be difficult to apply due to field conditions (Varzea *et al.*, 2002; van der Vossen & Walyaro, 2009). The use of coffee varieties that combines yield and quality with host resistance is considered the most appropriate and sustainable management strategy against diseases (Bettencourt & Rodrigues Jr., 1988; van der Vossen & Walyaro, 2009; van der Vossen *et al.*, 2015). The rapid outbreaks of CBD in Eastern Africa prompted some African countries as Kenya, Ethiopia and Tanzania, to carry out breeding programmes with the objective of combining yield with diseases resistance in coffee (Silva *et al.*, 2006; van der Vossen & Walyaro, 2009; van der Vossen *et al.*, 2015). In 1989, at CIFC (Centro de Investigação das Ferrugens do Cafeeiro)/ISA it was started a research line aiming to support breeding programs to CBD resistance in different coffee growing countries. Thousand progenies of coffee genotypes have been tested against an extended collection of *C. kahawae* isolates from different African regions, using hypocotyls tests, since previous work refers the existence of a correlation (r=0.73–0.80) between these tests and field resistance (van der Vossen *et al.* 1976). The hypocotyl prescreening tests allowed the selection of genotypes with high levels of resistance, such as some lines of Rume Sudan and derivatives of Timor Hybrid (HDT- a natural hybrid between *C. arabica* and *C. canephora*). Some HDT derivatives also show resistance to coffee leaf rust and nematodes (van der Vossen *et al.*, 1976; Silva *et al.*, 2006; Gichuru *et al.*, 2008). Progenies of HDT and advanced inbred lines of its cross to *C. arabica* cv. Caturra (referred to as variety Catimor), have been screened for CBD and leaf rust resistance and are used as donor parents in Kenya (Gichuru *et al.*, 2008).

1.2.1. Fungal infection process

Histological and ultrastructural studies revealed that, like most of *Colletotrichum* species (Latunde-Dada & Lucas, 2007; Vargas *et al.*, 2012; Kubo *et al.*, 2016), *C. kahawae* is a hemibiotrophic pathogen (Silva *et al.*, 2006 and references therein; Loureiro *et al.*, 2012). The

early stages of fungal development, in different coffee organs (hypocotyls, leaves and green berries), involves conidia germination and melanized appressoria differentiation (Chen *et al*., 2003, 2004; Loureiro *et al*., 2012; Silva *et al*., 2006 and references therein). In *C. kahawae*, melanized appressoria turgor pressure might play a major role in fungal penetration (Chen *et al*., 2004) which occurs directly by the epidermal cell walls through an infection peg. In susceptible tissues, the infection vesicle branches in intra- and inter-cellular hyphae. The pathogen first establishes a biotrophic interaction with the host and later switch to a destructive necrotrophic lifestyle. The biotrophic phase is repeated, as the fungus starts the colonization of new host cells. Therefore, it is possible to observe fungal hyphae in living and dead host cells simultaneously. Transmission electron microscope observations (Silva *et al.*, 2006; Loureiro *et al.*, 2012) showed that during the biotrophic stage the intracellular infection vesicles and hyphae remained external to the plant plasma membrane, which became invaginated around the fungus, and that host cells initially survive penetration and their cytoplasm seems to retain normal ultrastructure. In contrast, the necrotrophic stage is highly destructive, being characterize by an intense colonization of plant tissues associated with disruption of cell walls and cytoplasm collapse. This phase culminates with the appearance of disease symptoms and the production of acervuli which occurs within the epidermal cells with the subsequent rupture of cuticle and epidermis cell walls, and release of conidia.

1.2.2. Coffee resistance to CBD

Inheritance studies carried out in Kenya (van der Vossen *et al.*, 1980) concluded that coffee resistance to CBD appears to be controlled by major genes on three different loci, although the nature of such genes is still unknown. Nevertheless, the highly resistant variety Rume Sudan carries the dominant R- and the recessive K-genes, the moderately resistant variety K7 carries only the recessive K-gene and HDT carries one gene for CBD resistance on the T-locus with intermediate gene action. Additional evidence for oligogenic inheritance of CBD resistance was provided by the identification of molecular markers tightly linked to CBD resistance and the mapping of a locus carrying the gene in the variety Catimor (Gichuru *et al.*, 2008).
Cytological studies revealed that coffee resistance to *C. kahawae* is characterized by restricted fungal growth associated with: (i) cell wall modifications, such as accumulation of phenolic-like compounds followed by lignification, thickening and deposition of suberin; (ii) deployment of hypersensitive-like response (HR) identified by several ultrastructural changes,

including membrane breakdown, changes in the appearance of chloroplasts and the nucleus and coagulation of the cytoplasm; (iii) early deposition of callose around intracellular hyphae (Masaba & van der Vossen, 1982; Silva *et al.,* 2006 and references therein; Loureiro *et al*., 2012). Biochemical studies showed that peroxidase activity obtained from non-inoculated hypocotyls did not reveal differences between the resistant and susceptible varieties (Gichuru *et al.*, 1997).

1.3. The plant immune system

To escape from potential pathogens, plants evolved various constitutive and induced defence mechanisms leading to basic innate immunity. Performed barriers like wax layers, rigid cell walls, antimicrobial enzymes or secondary metabolites provide protection against pathogens that are not specialized to attack a specific host (Dangl & Jones, 2001). Nevertheless, pathogens can overcome these constitutive defensive layers and a second plant defence response can be elicited by two interconnected mechanisms namely, microbial/pathogen-associated molecular patterns (MAMP/PAMP)-triggered immunity (MTI/PTI - hereafter, referred to as PTI) and effector-triggered immunity (ETI) (Jones & Dangl, 2006; Muthamilarasan & Prasad, 2013). PTI and ETI mechanisms depend on receptors that can perceive the external or internal signals that will induce a downstream of similar defences that differs quantitatively and kinetically since ETI is regarded as an accelerated and amplified PTI response (Jones & Dangl, 2006; Sanabria *et al.*, 2010).

PTI correspond to a basal immunity that involves the recognition of conserved elicitors with signatures characteristic of a whole class of microbes (PAMP) or cell wall fragments, cutin monomers and peptides which result from the break of structural barriers that can function as endogenous elicitors called damage-associated molecular patterns (DAMPs). These DAMPs characteristically emerge in the apoplast, and serve as danger signals to induce innate immunity similar to MAMPs. PAMPS and DAMPs are perceived by a class of plasma-membrane-bound extracellular receptors called pattern recognition receptors (PRRs) (Boutrot & Zipfel, 2017). PRRs includes receptor-like kinases (RLK) and receptor-like proteins (RLP) that may form part of multi-component recognition complexes (Malinovsky *et al.*, 2014). RLK are transmembrane receptors with three domains; extracellular ligand-binding domain, a single transmembrane domain and an intracellular serine/threonine kinase domain, while RLP consists only of an extracellular and a transmembrane, lacking an intracellular domain (Boutrot & Zipfel, 2017).

ETI is induced when pathogens have successfully overcome the PTI strategies of defence and consequently promote pathogenesis by effector proteins. These effectors were identified originally not by their promotion of virulence but rather by their 'avirulence' activity. In fact, the effectors (Avr proteins from the pathogen) are recognized in a highly specific fashion by the products of host disease resistance (R) genes. Effectors can act as transcription factors or directly target host transcription factors and ultimately promoting the release of nutrients required for the survival of pathogen. Fungal and oomycete effectors can act either in the extracellular matrix or inside the host cell (Jones & Dangl, 2006). In this latter, effector recognition is achieved by intracellular receptors called nucleotide-binding leucine-rich receptors (NB-LRRs) through direct physical interaction between the receptor and effector or by an indirect interaction were the receptor monitors the integrity of that particular host target in order to detect manipulation of it by the effector (Mackey *et al.*, 2002, 2003; Muthamilarasan & Prasad, 2013).

The spatial and temporal patterns of cellular Ca^{2+} changes are termed *Ca^{2+} signatures* and are thought to elicit specific and appropriate response to a given signal (Reddy *et al.*, 2011; Cheval *et al.*, 2013; Ranty *et al.*, 2016). Ca^{2+} signatures are generated by the coordinated action of Ca^{2+} influx through various types of channels on the plasma membrane and through pumps and cotransporters on various organelles (Reddy *et al.*, 2011). Elevation of Ca^{2+} is perceived by calcium-binding proteins also called calcium sensors. These sensors belong to three main classes: calmodulin [including calmodulin-like proteins (CML)], calcium-dependent protein kinases (CPKs) and calcineurin B-like proteins (CBLs) (DeFalco *et al.*, 2010; Ranty *et al.*, 2016). Ca^{2+} binding sensors induces a conformational change of the protein that triggers either their association to downstream target proteins or a direct stimulation of the kinase activity (DeFalco *et al.*, 2010; Cheval *et al.*, 2013; Edel *et al.,* 2017). It is not yet fully elucidated how pathogen-induced Ca^{2+} signatures are translated to reprogram the transcriptome and altering defence responses (McCormack *et al.*, 2005; Reddy *et al.*, 2011; Ranty *et al.*, 2016).

Plant lipids and lipid metabolic pathways have been shown to be of crucial importance during a plant-pathogen interaction (Tayeh *et al.*, 2013). Lipids and lipid metabolites, released from membranes at the site of infection, function as signal molecules in the activation of plant defence responses. Phospholipid-signaling pathways are complex, inter-related, and involve numerous enzymes and substrates (Tayeh *et al.*, 2013). In particular, phospholipases A, that includes Patatin-like proteins (PTLs), hydrolyze phospholipids into the corresponding free fatty acid and lysophospholipid (such as lysoPC) (Canonne *et al.*, 2011). Fatty acid can be a precursor for oxylipin biosynthesis, and lysoPC may be involved in multiple cellular processes

like cytoplasmic acidification know to precede the activation of defence responses. PTLs have been implicated in plant defence signaling through jasmonic acid (JA) or oxylipin accumulation (Canonne *et al.*, 2011) and some reports indicates that PTLs can have a role in plant cell death through non-specific hydrolysis of membrane lipids (La Camera *et al.*, 2009). The synthesis of oxylipins is firstly catalyzed by lipoxygenases (LOXs) and LOX-derived hydroperoxides can be converted through different reactions of the LOX pathway, particularly by an allene oxide synthase (AOS) leading to JA (Shah, 2005; Tayeh *et al.*, 2013).

Phytohormones have been considered cellular signal molecules with key functions in the regulation of immune responses to microbial pathogens, insect herbivores, and beneficial microbes (Pieterse *et al.*, 2012). The plant hormone signaling pathways are interconnected in a complex network, enabling the plant to modulate efficient responses in order to adapt itself to different environmental conditions and to utilize its limited resources for growth and survival in a cost-efficient manner (Kunkel & Brooks, 2002; Pieterse *et al.*, 2012; Denancé *et al.*, 2013). However, pathogens evolved ingenious mechanisms to rewire the plant's hormone signaling network to suppress or evade host immunity (Kazan & Lyons, 2014). The successfulness of the role of phytohormones in plant defences depends on many factors, such as single hormone concentration, composition and timing of the hormone blend produced and hormone crosstalk (Verhage *et al.*, 2010; Pieterse *et al.*, 2012). The antagonist/synergetic crosstalk among different phytohormones [particularly salicylic acid (SA), JA and ethylene (ET)] seems to play a central role in the regulation of plant immune responses to the pathogens (Glazebrook, 2005; Bari & Jones, 2009). These defence responses are considered to be dependent on the pathogen lifestyle and the genetic constitution of the host (Pieterse *et al*,. 2009; Kazan & Lyons, 2014; Andolfo & Ercolano, 2015; Ma & Ma, 2016).

The activation of signaling cascades leads to the induction of a complex network of defence response, such as the hypersensitive reaction (HR), increase of oxidative enzymes (such as peroxidases) and cell wall modifications among others.

HR, a form of programmed plant cell death localized at the infection sites (Jones & Dangl, 2006), is particularly efficient halting the infection by biotrophic pathogens, since they depended on live cells to uptake nutrient. However for necrotrophic pathogens, induction of cell death may be used by the pathogen to aid in their invasion of the plant (Lam *et al.,* 2001). Typically, HR is associated with race-specific resistance involving a gene-for-gene interaction between microbe and plant (Christopher-Kozjan & Heath, 2003, Jones & Dangl, 2006). At cytological level, HR is characterized by cytoplasmic shrinkage, chromatin condensation, mitochondrial swelling, vacuolization and chloroplast disruption. Cell death appeared to

require an influx of extracellular calcium, protein kinase activity and protein synthesis (Christopher-Kozjan & Heath, 2003).

Oxidative enzymes, such as peroxidases (PODs) are a group of haem-containing glycosylated proteins that catalyse the oxidoreduction of various substrates using hydrogen peroxide (H_2O_2). Plant peroxidase superfamily can be divided in to three classes (I, II and III), based on the enzymatic primary structure. Class I consists of intracellular plant peroxidases in plants, bacteria and yeast, class II are extracellular peroxidases from fungi, and class III plant peroxidases (PODs) (EC 1.11.1.7) are secreted outside the cells or transported into vacuoles. The diversity of the substrates oxidized by PODs may explains why these proteins are implicated in a broad range of physiological processes, but specific functions of individual PODs have been difficult to define because of their low substrate specificity *in vitro* and the presence of many isoenzymes (Hiraga, 2001). Due to the importance of PODs in multiple physiological processes, they have been subject of several reviews (Cao *et al.,* 2016; Hiraga, 2001; Almagro *et al.*, 2009). Among the most important roles of PODs are production/scavenging of reactive oxygen species (ROS), cross-linking of cell wall structural proteins and lignification (Hiraga, 2001; Almagro *et al.*, 2009; O'Brien *et al.*, 2012; Ge *et al.*, 2013; Cao *et al.*, 2016; Survila *et al.*, 2016). In plants, polyphenol oxidases (PPOs) comprised of two different enzymes; cresolase (E.C. 1.14.18.1) and catalase (EC 1.10.3.1). PPOs that are responsible for the oxidation of monophenols into *o*-diphenols and *o*-diphenols to *o*-diquinones respectively (Vaughn & Duke 1984; Yoruk & Marshall 2003; Aniszewski *et al.* 2008; Li *et al.* 2017). Quinones are highly reactive compounds that can spontaneously complex to various types of molecules accelerating the cellular death of infected cells, generating a toxic environment to the pathogen, reducing the protein nutritional value and being able to react with other phenolic compounds leading to additional barriers that can prevent pathogen penetration (Melo *et al.*, 2006). While the physiological function of PPO in many plants is unresolved, in some species there is strong evidence for a role of PPO in defence against insects as well as pathogens (Constabel *et al.*, 2000; Haruta *et al.*, 2001; Li & Steffens 2002; Mohammadi & Kazemi, 2002; Wang & Constabel, 2004). PPO is a plastid enzyme been found loosely attached to the luminal side of the thylakoid membranes (Vaughn & Duke 1984), while PPO substrate candidates (phenolic acids and flavonoids) are reported to be mainly localized in the vacuole and other subcellular compartments (Achnine *et al.*, 2004; Chen *et al.*, 2006; Tran *et al.*, 2012). The proposed model for PPO activity states that enzyme and substrate come into contact after cells are wounded or disrupted by pathogens (Mohammadi & Kazemi, 2002).

The plant cell wall is a complex and dynamic network of polysaccharides and glycoproteins that responds to external or internal cellular signals. Cell wall polysaccharides (e.g., 1,4-β-D-glucan, β-(1-3)-glucan, xyloglucan, xylans, mannans and pectic polysaccharides homogalacturonan, xylogalacturonan) have many different composition and structure that can be modified during plant development and in response to different stimulus (Pogorelko *et al.* 2013). *MUR4* (UDP-arabinose 4-epimerase 1) and *PME41* (pectinesterase/pectinesterase inhibitor 41) genes are related with polysaccharides biosynthesis at cell wall level. MUR4 catalyzes the 4-epimerization of UDP-D-xylose to UDP-L-arabinose, the nucleotide sugar used by glycosyltransferases in the arabinosylation of cell wall polysaccharides, and wall-resident proteoglycans (Burget *et al.*, 2003). One example is the pectic arabinan, a major l-arabinose-containing molecule in the cell walls of many plants, which undergoes hydrolysis by glycoside hydrolases (Kotake *et al.*, 2016). Furthermore, L-arabinose is an essential component of hydroxyproline--rich glycoproteins (HRGPs) and arabinogalactan proteins (Stafstrom & Staehelin, 1986), that play a structural role in strengthening the cell wall and are expressed in response to pathogen attack (Showalter, 1993). Changes in arabinosylated glycans can lead to alterations in covalent cross-linking between different cell wall polymers. For instance, covalent ester-ether bridges between polysaccharides and lignins are formed by ferulic acids on arabinoxylans in wheat straw (Iivama *et al.*, 1990), and these ferulic acids are esterified to pectins via arabinose residues in spinach and sugar beet (Lamb *et al.*, 1990). It has been proposed that pectin is synthesized and modified as it moves through the Golgi and is transported via vesicles to the cell wall (Atmodjo *et al.*, 2013). According to this model an increasingly complex pectin structure, is produced by a series of glycosyltransferase that consecutively add sugars from nucleotide-sugar substrates onto growing polymer chains as they move through the cis-, medial-, and trans-Golgi apparatus (Caffall & Mohnen, 2009; Atmodjo *et al.*, 2013). Pectins are secreted in the apoplast in a highly methylesterified form by the action of pectin methyltransferases (PMTs) but after its deposition, pectin methylesterases (PMEs) selectively remove the methyl groups (Caffall & Mohnen, 2009; Lionetti *et al.*, 2012). The degree and pattern of the de-esterification affects the mechanical properties of the wall matrix by altering the affinity of pectins for calcium ions and pectin–cellulose assembly, for example. The degree and pattern of the de-esterification turns pectin more susceptible to microbial pectic enzymes to other cell wall degrading enzymes (CWDEs) (Lionetti *et al.*, 2012), but also affects the action of polygalacturonases and favours the accumulation of pectin breakdown fragments like oligogalacturonides (OGs), that has a known elicitor activity (Ferrari *et al.*, 2013; Benedetti *et al.*, 2015)

1.4 Objectives and thesis outline

Despite the knowledge advances gathered so far in the coffee - *C. kahawae* interaction, there is still much to learn about the biochemical and molecular basis of host resistance. Therefore, based on the information that the coffee variety Catimor 88 has been successfully used as resistance source to *C. kahawae* in Kenyan breeding programmes, this research aims to characterize its resistance to the isolate Que 2 (a representative isolate from Kenya), combining cytological, biochemical and molecular tools. The susceptible variety Caturra was used comparatively.

To accomplish the above-mentioned aim, we intend to fulfil the following specific research objectives:

i) to quantify the fungal growth and early host responses, with emphasis in the hypersensitive-like cell death (HR), accumulation of phenolic-like compounds and cell wall modifications by light microscopy;

ii) to evaluate oxidase activities by spectrophotometry, electrophoresis and histochemistry;

iii) to study, through quantitative real-time PCR, the expression profiles of genes putatively involved in pathogen recognition, signaling, phytohormones pathways, oxidases and cell wall modifications.

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Chapter 2

A First insight into the involvement of phytohormones pathways in coffee resistance and susceptibility to *Colletotrichum kahawae*

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2. A First insight into the involvement of phytohormones pathways in coffee resistance and susceptibility to *Colletotrichum kahawae*

Abstract

Understanding the molecular mechanisms underlying coffee-pathogen interactions are of key importance to aid disease resistance breeding efforts. In this work, the expression of genes involved in salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) pathways were studied in hypocotyls of two coffee varieties challenged with the hemibiotrophic fungus *Colletotrichum kahawae*, the causal agent of Coffee Berry Disease. Based on a cytological analysis, key time points of the infection process were selected and qPCR was used to evaluate the expression of phytohormones biosynthesis, reception, and responsive-related genes.

The resistance to *C. kahawae* was characterized by restricted fungal growth associated with early accumulation of phenolic compounds in the cell walls and cytoplasmic contents and deployment of hypersensitive reaction. Similar responses were detected in the susceptible variety, but in a significantly lower percentage of infection sites and with no apparent effect on disease development. Gene expression analysis suggests a more relevant involvement of JA and ET phytohormones than SA in this pathosystem. An earlier and stronger activation of the JA pathway observed in the resistant variety when compared with the susceptible one, seems to be responsible for the successful activation of defence responses and inhibition of fungal growth. For the ET pathway, the down or non-regulation of ET receptors in the resistant variety, together with a moderate expression of the responsive-related gene *ERF1*, indicates that this phytohormone may be related to other functions besides the resistance response. However, in the susceptible variety, the stronger activation of *ERF1* gene at the beginning of the necrotrophic phase, suggests the involvement of ET in tissue senescence. As far as we know, this is the first attempt to unveil the role of phytohormones in coffee-*C. kahawae* interactions, thus contributing to deepen our understanding on the complex mechanisms of plant signaling and defence.

2.1. Introduction

Coffee Berry Disease (CBD), caused by the hemibiotrophic fungus *Colletotrichum kahawae* J.M. Waller & P.D. Bridge, is a major constraint of Arabica coffee production in Africa. This disease may cause up to 50-80% of crop losses, in years of severe epidemics if control measures are not applied (van der Vossen & Walyaro, 2009). Since the first report in 1922 in Kenya (McDonald, 1926), CBD is still restricted to Africa but there are major concerns about the risk of its introduction into Latin America and Asia (Silva *et al.,* 2006; van der Vossen & Walyaro, 2009). *C. kahawae* infects several coffee organs, but major losses result from the infection of green berries. The outbreak of the disease with visible symptoms occurs during the expanding stage of berry development, producing dark, sunken anthracnose-like lesions on the green pulp (Firman & Waller, 1977; Hindorf & Omondi, 2011). Although the application of fungicides can provide adequate control, the use of coffee resistant varieties is the most appropriate and sustainable management strategy against this disease.

Inheritance studies carried out in Kenya by van der Vossen and Walyaro (1980) and recent molecular studies (Omondi & Pinard, 2006; Gichuru *et al.,* 2008) provided evidences that coffee resistance to *C. kahawae* appears to be controlled by major genes in different loci. Cytological and biochemical studies revealed that coffee resistance to *C. kahawae* is characterized by restricted fungal growth associated with several host responses, such as hypersensitive-like cell death (HR), formation of cork barriers, callose deposition around intracellular hyphae, accumulation of phenolic compounds (flavonoids and hydroxycinnamic acid derivatives), lignification of host cell walls and increased activity of oxidative enzymes, such as peroxidases (Masaba & van der Vossen, 1982; Gichuru, 1997, 2007; Silva *et al.,* 2006; Loureiro *et al.,* 2012, 2013). More recently, differentially expressed genes involved in recognition, signaling and defence responses of coffee to *C. kahawae* have been identified (Figueiredo *et al.,* 2013; Diniz *et al.,* 2014, 2015).

Plant growth and responses to environmental cues are largely governed by phytohormones. Recent research indicates that an antagonist/synergetic crosstalk among different phytohormones [particularly salicylic acid (SA), jasmonic acid (JA) and ethylene (ET)] play a central role in the regulation of plant immune responses to the pathogen (Glazebrook, 2005; Bari & Jones, 2009). These defence responses are considered to be dependent on the pathogen lifestyle and the genetic constitution of the host (Pieterse *et al.,* 2009; Kazan & Lyons, 2014; Andolfo & Ercolano, 2015; Ma & Ma, 2016).

In plants, SA can be synthesized via two distinct enzymatic pathways that require the primary metabolite chorismate: phenylalanine ammonia-lyase (PAL)-mediated phenylalanine and

isochorismate synthase (ICS)-mediated isochorismate. The NPR1 (non-expressor of pathogenesis-related genes 1) represents a key node in signaling downstream from SA (Fig. S2.1a) (Yan & Dong, 2014). In the absence of SA or pathogen challenge, NPR1 is inactive in cytoplasm as an oligomer. Upon induction, SA accumulation is promoted and NPR1 monomer is released to enter the nucleus where it activates defence gene transcription, such as the *PR1* gene (An & Mou, 2011).

JA biosynthesis starts with the release of α-linolenic acid (α-LA) from membrane lipids and its oxygenation in the chloroplast, followed by the sequential action of allene oxide synthase (AOS) and allene oxide cyclase (AOC), resulting in the synthesis of 12-oxophytodienoic acid (OPDA) (Fig. S2.1b) (Pieterse *et al.,* 2012; Figueiredo *et al.,* 2015). OPDA migrates to the peroxisome to be reduced by oxophytodienoate redutase 3 (OPR3) and undergo several rounds of β-oxidation to form JA. Then, JA is exported from the peroxisome to cytosol for conjugation to the L-isoleucine (Ile) by jasmonate-resistant 1 (JAR1) resulting in the endogenous bioactive form of JA-Ile (Staswick & Tiryaki, 2004; Fonseca *et al.,* 2009). JA-Ile then interacts with coronatine insensitive 1 (COI1), an F-box protein which acts as a JA receptor, and targets jasmonate negative regulators [like jasmonate zim domain proteins (JAZ)] for degradation, promoting JA-induced gene transcription such as of *PR10* (Figueiredo *et al.,* 2015; Ahmad *et al.,* 2016).

Ethylene is produced from methionine via S-adenosyl-L-methionine (SAM) being the last two biosynthetic steps catalysed by aminocyclopropane-1-carboxylic synthase (ACS) and ACC oxidase (ACO) (Fig. S2.1c) (Yang & Hoffman, 1984; Kende, 1989; Wang *et al.,* 2002). In the presence of ET, the receptor ethylene resistant 1 (ETR1) binds to the hormone switching off the constitutive triple response 1 (CTR1). Consequently, desphosphorylation of C-terminal end of ethylene insensitive 2 (EIN2) is promoted and the repression of the transcriptional signaling cascade is unlocked (Merchante *et al.,* 2013).

Despite the recent advances achieved on the complex regulation of phytohormones network in plants under different environmental conditions (Wasternack, 2014; Das *et al.,* 2015; Janda & Ruelland, 2015), this knowledge is still very scarce in plant-*Colletotrichum* spp. pathosystems, particularly in the coffee-*C. kahawae* interaction. The aim of this study was to elucidate the possible involvement of the phytohormones SA, JA and ET in the responses of coffee plants resistant (variety Catimor 88) and susceptible (variety Caturra) to *C. kahawae.* Based on a cytological analysis of the fungal growth and the associated host responses, time points of the infection process were chosen to evaluate the expression of phytohormones biosynthesis, reception and responsive-related genes by quantitative real-time PCR (qPCR).

2.2. Material and Methods

2.2.1. Plant material and inoculation

Experimental assays were conducted on coffee hypocotyls, since previous studies shown a correlation between the pre-selection test on hypocotyls and mature plant resistance in the field (r= 0.73 – 0.80) (van der Vossen *et al.,* 1976).

For this study, the varieties Catimor 88 (Timor hybrid derivative, which exhibit field resistance to *C. kahawae* breeding programmes in Kenya) and Caturra (CIFC 19/1 – *Coffea arabica* L.) were used as resistant and susceptible varieties to *C. kahawae* isolate Que2 (from Kenya), respectively. Coffee seeds were sown in a mixture of soil:peat:sand (1:1:1) and grown under greenhouse conditions with average temperatures between 16ºC and 28ºC (minimum and maximum temperatures, respectively) during 8 weeks.

Conidia of *C. kahawae* isolate Que2, retrieved from a *C. kahawae* collection maintained at CIFC/ISA, were produced after 7 days at 22°C on malt extract agar (MEA) (Rodrigues *et al.,* 1991). Hypocotyls of Catimor 88 and Caturra, were inoculated according to the technique described by van der Vossen *et al.,* (1976) with slight modifications. Briefly, hypocotyls were vertically placed on plastic trays containing a wet nylon sponge and sprayed with a conidia suspension ($3x10^6$/ml). The trays were then covered with a plastic bag to simulate a humid chamber and were kept in a Phytotron750 E at 22ºC. For the first 24 hours post inoculation (hpi), the trays were kept in the dark and afterwards a 12 hours photoperiod was established for the remaining time-course of the experiment. Non-inoculated hypocotyls sprayed with water were kept in the same conditions as the inoculated hypocotyls and used as control.

2.2.2. Light microscopic observation in fresh tissues

Conidial germination and appressorial differentiation were observed on hypocotyl pieces ($5cm^2$) at 3, 6, 9, 12, 15, 18 and 24hpi, as previously described (Silva *et al.,* 1999). The hypocotyl pieces were painted with transparent nail polish on the inoculated surface to recreate a tissue surface replica. Once dried, the nail polish was removed, stained and mounted in lactophenol cotton blue. For each experiment, a minimum of six microscope fields, each containing 100 conidia and/or differentiated appressoria on the surface of hypocotyls, were used. To evaluate fungal post-penetration growth stages at 24, 48 and 72hpi, cross sections of inoculated hypocotyl fragments made with a freezing microtome (CM1850, Leica), were

stained and mounted in lactophenol cotton blue (Silva *et al.,* 1999; Loureiro *et al.,* 2012). Hyphal length inside hypocotyl tissues were estimated with the aid of a micrometric eyepiece. To detect autofluorescent cells, cross sections of non-inoculated (control) and inoculated tissues were placed in 0.07M pH 8.9 phosphate solution (K_2HPO_4) for 5 min, and mounted in the same solution (Silva *et al.,* 2002; Loureiro *et al.,* 2012). Autofluorescence under epifluorescence blue light is thought to indicate the presence of phenolic-like compounds and cytoplasmic autofluorescence and/or browning is frequently associated with plant cell death (Bennett *et al.,* 1996; Heath, 1998). Observations were made using light microscopes (LeitzDialux 20 and DM-2500, Leica) equipped with a mercury bulb HB 100W blue light (excitation filter BP 450 and 490; barrier filter LP 515).

Data on pre- and post-penetration fungal growth stages and host cell responses are presented as the combined values of three experiments. Fungal growth inside host tissues and plant responses were recorded from 75 to 100 infection sites per experiment at three time points (24, 48 and 72hpi).

2.2.3. RNA extraction and cDNA synthesis

Hypocotyls (inoculated and non-inoculated) were harvested at 6, 12, 24, 48 and 72hpi, immediately frozen in liquid nitrogen and stored at -80ºC prior to RNA extraction. Three biological replicates, each representing a pool of 15 hypocotyls, were used.

Total RNA was extracted with the Spectrum Plant Total RNA kit (Sigma-Aldrich), according to the manufacturer's instructions. Residual genomic DNA was digested with DNase I (On-column DNase I Digestion Set, Sigma-Aldrich). RNA purity and concentration was measured at 260/280 nm and 260/230 nm using a spectrophotometer (NanoDrop 1000, Thermo Scientific). RNA integrity was verified by electrophoresis in 1% agarose gel. Genomic DNA (gDNA) contamination was checked by qPCR analysis on the crude RNA (Vandesompele *et al.,* 2002). Complementary DNA (cDNA) was synthesized from 2.5µg of total RNA using RevertAid®H Minus Reverse Transcriptase (Fermentas) anchored with Oligo$(dT)_{23}$primer (Fermentas), according to manufacturer's instructions.

2.2.4. Primer design

Within the targeted phytohormone pathways, sixteen genes related with biosynthesis, reception and responsiveness (Merchante *et al.,* 2013; Kumar, 2014; Figueiredo *et al.,* 2015) were selected for expression analysis in *Coffea* spp. after *C. kahawae* challenge (Table 2.1). For SA, the following genes were included: Isochlorismate synthase 2 (*ICS2*); Phenylalanine ammonia-lyase (*PAL)*; Non-expressor of pathogenesis-related 1 (*NPR1)*; Pathogenesis-related (*PR1*). For JA the following genes were included: 12-oxoplytodienotae reductase 1-like (*OPR3)*; Coronatine insensitive 1 (*COI1)*; Pathogenesis-related 10 (*PR10*). For ET the following genes were included: 1-aminocyclepropane-1-carboxylic acid synthase 5 (*ACS5)*; 1-aminocyclepropane-1-carboxylic acid oxidase 2 (*ACO2)*; Ethylene resistant 1 (*ETR1)*; Ethylene insensitive 2 (*EIN2)*; Constitutive triple response 1 (*CTR1)*; Ethylene-responsive factor 1 (*ERF1*). With the exception of *PAL* and *PR10* genes, that were previously described in coffee (Ramiro *et al.,* 2009), the remaining fourteen genes were retrieved from a coffee RNA-seq database (Fino *et al.,* 2015) as being orthologous of previously described genes in *Arabidopsis thaliana* (TAIR database: www.arabidopsis.org). Tubulin beta-9 (*β-Tub9*)/ ribosomal protein S24 (*S24)* and Insuline Degrading Enzyme (*IDE*)/*S24* were used as reference genes for susceptible and resistant varieties samples, respectively (Figueiredo *et al.,* 2013). Coffee specific primers (Table 2.1) were designed with PrimerSelect version 5.0 (DNAStar, Inc.) using the following parameters: amplicon length 70 and 200 bp; size between 17 and 22 bp; annealing temperature (Ta) between 58 and 62ºC and GC content± 50%.

Table 2. 1 - Primer sequences for qPCR analysis of target and reference genes

Gene	Coffee source name	Name	Primer sequence (5'-3')	Primer length (bp)	Amplicon length (bp)	Ta (ºC)	Tm (ºC)	PCR efficiency
ICS2	Scaffold21359	Isochorismate synthase 2	Fw: TGCCATAGTACGAGAAAACA	20	124	60	79.0	94
			Rev: CCCAGAAAATCGACCATAAA	20				
PAL[a]	CaPAL F 13097	Phenylalanine ammonia-lyase	Fw: GCAGGTCCTACTCATTGTACAAG	23	166	60	82.0	89
			Rev: CCATTCCACTCTTTCAAACAATCC	24				
NPR1	Scaffold33187	Non-expressor of PR1	Fw: AGGGCATTGGATTCTGACGA	20	126	60	81.5	88
			Rev: CTCTGTTGTGGTCTTTGCGT	20				
PR1	Scaffold170607	Pathogenesis-related 1	Fw: GCCCGTAAAGTCACCTGT	18	177	60	86.5	91
			Rev: AACTACGCTGCCAAAATC	18				
OPR3	Scaffold2739	12-oxoplytodienotae reductase 1-like	Fw: ATAACTCCCCACCTTCCAAC	20	198	58	81.5	91
			Rev: ACAGCCTTATCCCAACTCTAT	22				
COI1	Scaffold40077	Coronatine insensitive 1	Fw: CTTAGCATCACCACCCACC	19	157	62	81.5	93
			Rev: TCCGATCCCCCATACCAAC	19				
PR10[b]	CF589103	Pathogenesis-related 10	Fw: GCCACCATCCTTGAAGAGAA	20	151	55	80.0	99
			Rev: CAACTCTCTGCTTGGCAGTCT	21				
ACS5	Scaffold82864	1-aminocyclepropane-1-carboxylic acid synthase 5	Fw: AGGGCGTCCTGGTCACTAA	19	148	60	83.0	90
			Rev: CTCGGCGAGCTAAAAACTGT	20				
ACO2	Scaffold57328	1-aminocyclepropane-1-carboxylic acid oxidase 2	Fw: AAAGTCAGCAATTACCCTCCA	21	144	58	87.0	93
			Rev: ATCCACCCATTCACCATCCT	20				
ETR1	Scaffold776	Ethylene resistant 1	Fw: GCCCCCAAGATATTCCTAAG	20	92	60	79.5	89
			Rev: TGCAAGACCAAGACCACTAC	20				
EIN2	Scaffold3828	Ethylene insensitive 2	Fw: GTTACTTCTCCAAAACCTACT	21	134	60	78.5	93
			Rev: TCCCATTTACCACTCTTATCT	21				
CTR1	Scaffold16054	Constitutive triple response 1	Fw: GCAGCTGTGGGTTTCAAGG	19	162	60	83.0	90
			Rev: AGTGGGGGAGGGTTTAGTC	19				
ERF1	C312112	Ethylene-responsive factor 1	Fw: TGGCTGGGCACATTTGAC	18	85	58	84.0	90
			Rev: GGATTGCTGCTTGACCTC	18				
IDE[c]	isotig10635	Insuline Degrading Enzyme	Fw: TGATCTAAGCTGGTGGAAAGC	21	91	55	76.3	99
			Rev: TCAGGTGCATCAGGATGATT	20				
S24[c]	SNG-U349723*	Ribosomal protein S24	Fw: GCCCAAATATCGGCTTATCA	20	92	60	7.6	95
			Rev: TCTTCTTGGCCCTGTTCTTC	20				
β-Tub9[c]	isotig08544	Tubulin beta-9	Fw: ACCCTCCAGCAAACTGATGA	20	100	55	77.3	92
			Rev: AGGATGCCACTGCTGATGAT	20				

bp – base pairs, Ta – Annealing temperature, Tm – Melting temperature

* Unigene accession number according to the SOL Genomics Network.

[a] Fernandez *et al.,* (unpublished).

[b] Ramiro *et al.,* 2009.

[c] Figueiredo *et al.,* 2013.

2.2.5. Quantitative real-time PCR

The qPCR experiments were carried out using SYBR Green Supermix (Bio-Rad) in an iQ5 real-time thermal cycler (Bio-Rad). Each 25µl reaction comprised 4µl cDNA template (2.5µg/µl), 12.5µl SYBR Green Supermix (Bio-Rad), 0.4µl of each primer (10µM) and 0.7µl of sterile distilled water. Thermal cycling started with a denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15s and annealing at the respective temperature for each gene (Table 2.1) for 30s. Each set of reactions included a negative control

with no template. The amplification efficiency for each gene of interest was determined using the LinRegPCR version 2013.0. Dissociation curves (Fig. S2.2) and agarose gel electrophoresis were used to analyse non-specific PCR products. Three biological replicates and two technical replicates were used for each sample. Relative gene expression (fold change) was calculated according to Hellemans *et al.* (2007). The gene expression data were further visualized using the software MeV viewer (www.tm4. org).

2.2.6. Statistical analysis

For statistical analysis of cytological data, Student's *t*-test was applied using IBM®SPSS® Statistics version 20.0 (SPSS Inc.) software and arcsine-transformed percentages were used where appropriate. Statistical significance ($p \leq 0.05$) of gene expression between the two coffee varieties was determined by the non-parametric Mann–Whitney *U* test using IBM®SPSS® Statistics version 20.0 (SPSS Inc.) software.

2.3. Results

2.3.1. Fungal infection and host responses

The development of pre-penetration fungal growth stages of *C. kahawae* was similar in both resistant and susceptible varieties (Fig. 2.1; Table 2.2). Conidial germination and appressorial differentiation were initiated at 3 and 6hpi, respectively. At 12hpi, 42% of the appressoria were melanized reaching 89-94% at 24hpi (Fig. 2.2a). In both coffee varieties, the melanized appressoria began to penetrate the epidermal cells with the formation of a globose infection vesicle, at 48hpi (Fig. 2.2b). Hyphae developed from the vesicles grew either intra- and intercellularly colonizing epidermal and cortex cells. In the susceptible variety, the fungus pursued its growth feeding on living host cells (biotrophy) before switching to necrotrophy (72hpi) in the majority of the infection sites (about 90%) (Fig. 2.2c and d). Resistance was characterized by restricted fungal growth (fungal hyphae were more frequently confined to the epidermal cells or occasionally to those of the first layer of the cortex cells), as confirmed by the evaluation of hyphal length (Fig. 2.2e and f; Table 2.3). The entanglement of fungal hyphae

originating from different infection sites, together with the increasing number of necrotic host cells in susceptible tissues, did not allow the quantification of the hyphal length beyond 72hpi.

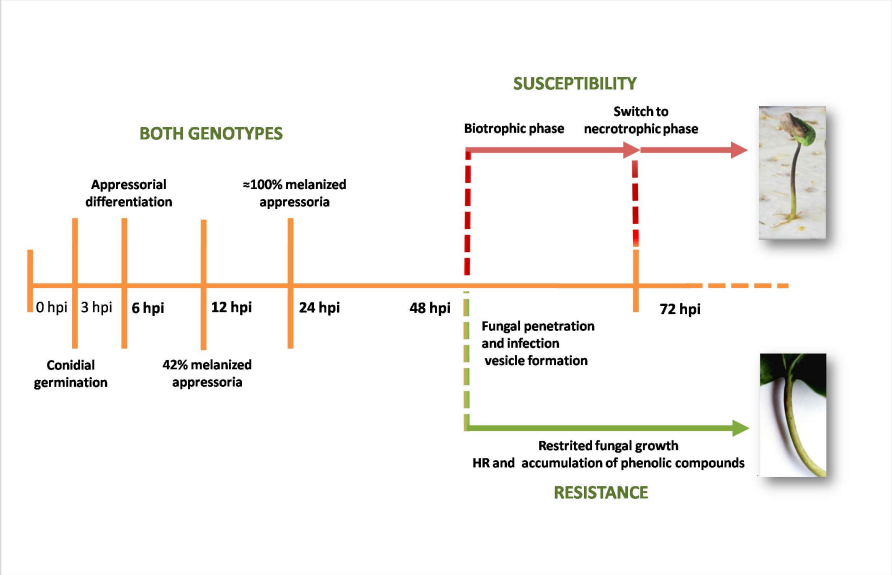

Figure 2.1 -Time-course of *Colletotrichum kahawae* (isolate Que2) infection in hypocotyls of resistant (Catimor 88) and susceptible (Caturra) coffee varieties. 3hpi and 6hpi – beginning of conidia germination and appressoria differentiation, respectively; 12hpi – 42% of melanized appressoria; 24hpi – almost 100% of melanized appressoria; 48hpi - fungal penetration and biotrophic growth; 72hpi – switch to necrotrophy (susceptible variety) and restriction of fungal growth (resistant variety). Times post inoculation (bold) were selected to collect samples for gene expression studies by qPCR.

Table 2. 2 - Percentage of conidial germination and appressorial differentiation of *Colletotrichum kahawae* on hypocotyls of resistant and susceptible coffee varieties, at different hours post inoculation.

Hours after inoculation	Coffee varieties	Germinated conidia (%) (x ± SD)	t test*	Appressoria** (%) (x ± SD)	t test*	Melanized appressoria (%) (x ± SD)	t test*
3 h	Catimor 88 (R)	3±3	0.15 ^{ns}	0	-	0	
	Caturra (S)	4±4		0		0	
6h	Catimor 88 (R)	16±13	0.90$^{n.s}$	43±26	0,32 ^{ns}	1±1	0.37$^{n.s}$
	Caturra (S)	16±33		46±33		2±2	
9h	Catimor 88 (R)	26±9	0.35^{ns}	65±16	0.92^{ns}	17±12	0.06$^{n.s}$
	Caturra (S)	29±15		65±36		9±9	
12h	Catimor 88 (R)	36±33	0.32^{ns}	69±18	0.34^{ns}	42±11	0.92$^{n.s}$
	Caturra (S)	33±26		76±27		42±31	
15h	Catimor 88 (R)	50±18	0.34^{ns}	78±11	0.94^{ns}	49±17	0.34$^{n.s}$
	Caturra (S)	57±17		79±8		56±26	
18h	Catimor 88 (R)	61±18	0.36^{ns}	87±13	0.95^{ns}	72±20	0.95$^{n.s}$
	Caturra (S)	65±24		88±10		71±23	
24h	Catimor 88 (R)	71±22	0.96^{ns}	89±14	1,16 ^{ns}	89±11	1.16 ^{ns}
	Caturra (S)	72±20		94±6		94±6	

R=Resistant; S=Susceptible; X ± SD = mean ± standard deviation; * Student's *t* test (ns - non significative); ** Total of appressoria (non melanized and melanized)

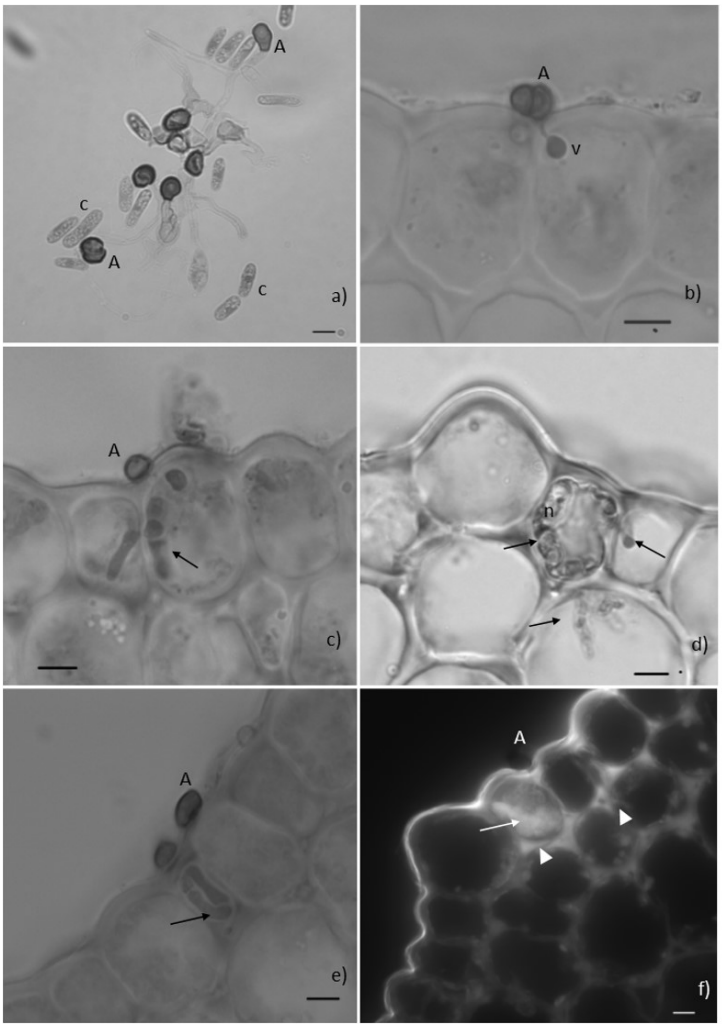

Figure 2.2 - Fungal pre- and post-penetration growth stages and host responses. Light microscope observations, cotton blue lactophenol staining (2.2a-e) and epifluorescence test under blue light (2.2f). Fig 2.2a. Conidia (C) germination and formation of melanized appressoria (A) on the surface of a resistant hypocotyl, 24 hours post inoculation (hpi). Fig 2.2b. Infection site showing a melanized appressorium (A) and an infection vesicle (v) in the epidermal cell of the resistant hypocothyl, 48hpi. Fig 2.2c. Infection site showing a melanized appressorium (A) and hyphae inside two adjacent epidermal cells of the susceptible hypocotyl (arrow), 48hpi. Fig 2.2d. Fungal hyphae (arrows) in living and in necrotized (n) cells of the susceptible hypocotyl, 72hpi. Fig 2.2e. Infection site showing a melanized appressorium (A) and intracellular hyphae (arrow) confined to the epidermal cell of the resistant hypocotyl, 72hpi. Fig 2.2f. Infection site showing an appressorium (A) associated with autofluorescence of the cytoplasmic content of one epidermal cell (HR-like). Note that the walls of this cell and of adjacent epidermal and cortex cells are also autofluorescent (bars=10µm).

Table 2. 3 - Evaluation of fungal growth in coffee hypocotyls of resistant and susceptible coffee varieties after challenge with *Colletotrichum kahawae,* at different hours post inoculation.

	Hyphal length (µm)/infection site in hypocotyls of coffee varieties		
Hours post inoculation	Catimor 88 (R) x± SD	Caturra (S) x± SD	*t* test*
24	0	0	–
48	3.94±2.11	8.43±8.22	1.49^{ns}
72	12.76±8.81	42.6±32.52	4.25^{***}

R=Resistant; S=Susceptible; X ± SD, mean ± standard deviation; * Student's *t*-test (ns - non significative; ***p≤0.001)

In the resistant variety, the first cytological changes were displayed in the epidermal cells at 24hpi and corresponded to: (i) accumulation of phenolic-like compounds [indicated by autofluorescence (AF) in cell walls only or in cell walls plus the cytoplasmic content]; (ii) deployment of HR (monitored by the AF and/or browning of the cytoplasmic contents) (Fig. 2.2f and 2.3). During the time course of the infection, these responses spread to adjacent cells of the epidermis and of the first layer of cortex cells. Similar responses were detected in the susceptible variety, but in a significantly lower percentage of infection sites (24hpi: 2%, 48hpi: 4%, 72hpi: 10%) comparatively to the resistant variety (24hpi: 6%, 48hpi: 26%, 72hpi: 56%) (Fig. 2.3). In these infection sites the fungus stopped its growth at the stage of appressoria or infection vesicle.

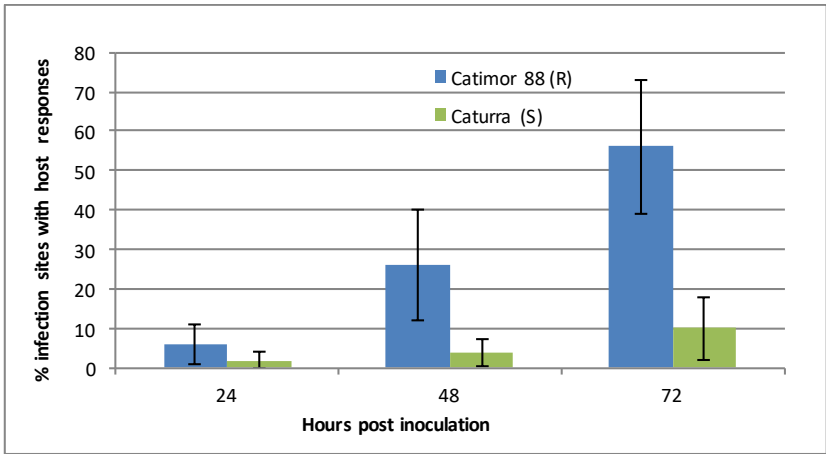

Figure 2.3 - Percentage of infection sites with host responses. Accumulation of phenolic-like compounds in the cell walls only, or in both the cell walls and the cytoplasmic contents and deployment of hypersensitive-like cell death (HR) induced by *C. kahawae* in hypocotyls of coffee varieties Catimor 88 (R-resistant) and Caturra (S-susceptible), at different hours post inoculation. The average percentages were significantly higher in the resistant than in the susceptible coffee variety at 24hpi (t = 2.52; $p \leq 0.05$), 48hpi (t = 4.83; $p \leq 0.001$) and 72hpi (t = 6.69; $p \leq 0.001$).

At 5-6 days after inoculation, resistant hypocotyls exhibited scab lesions or, occasionally absence of macroscopic symptoms, whereas susceptible hypocotyls showed typical dark sunken lesions with sporulation (Fig. 2.1).

2.3.2. Expression of genes from SA, JA and ET pathways

Phytohormone pathway induction during *Coffea* spp.-*C. kahawae* interaction was monitored by the expression analysis of sixteen related genes. For a global perspective of the relative expression levels of SA, JA and ET pathway-related genes in the resistant and susceptible varieties along the infection process, a heatmap analysis was performed (Fig. 2.4).

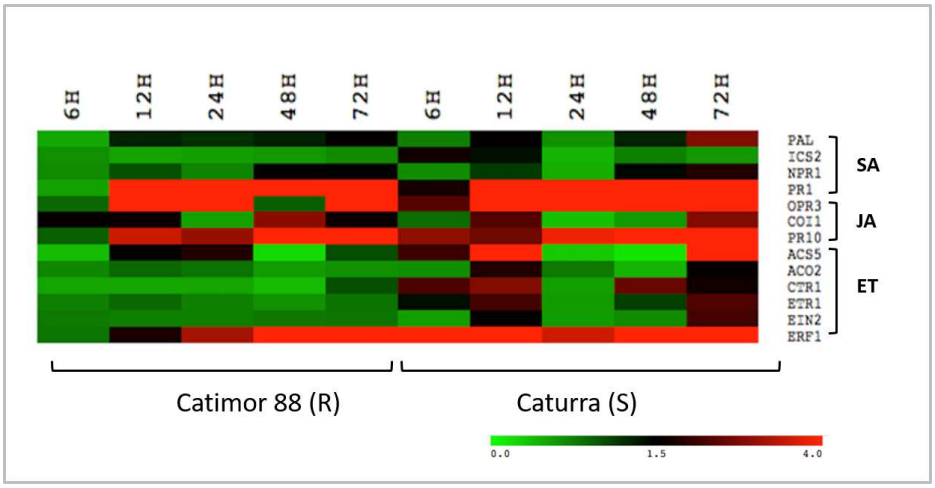

Figure 2.4 - Expression analysis of SA, JA and ET pathway related genes in non-inoculated hypocotyls of Catimor 88 (R-resistant) and Caturra (S-susceptible) vs inoculated hypocotyls with *C. kahawae*. Heatmap was colored according to the log2 ratio of expression, where green indicates lower expression, red indicates higher expression and black indicates no expression (see the color scale); in columns are the time points studied (6, 12, 24, 48 and 72hpi) and in the rows the genes analyzed.

The expression patterns of SA pathway-related genes were quite similar for both coffee varieties; being SA biosynthesis and receptor-related genes (*ICS2*, *PAL*, *NPR1*) mostly non-regulated, while *PR1* gene was up-regulated from 12hpi onwards (Fig. 2.4 and Fig. S2.3). On the contrary, the expression level of genes from JA and ET pathways showed differences between the two varieties (Fig. 2.4).

The activation of the JA pathway was observed in both coffee varieties, but differently in timing and magnitude (Fig. 2.5). In the susceptible variety, the biosynthesis-related gene *OPR3* was up-regulated in all time points showing the highest level of expression at 72hpi (25.5±14.9). JA-Ile receptor gene *COI1* was up-regulated at 12hpi (2.3±0.14) and 72hpi (2.7±0.9), while the responsive–related gene *PR10* showed the maximum value of expression at 72hpi (70.3±3.3). In the resistant variety, the up-regulation of the biosynthesis-related gene *OPR3* was observed at 12-24hpi (12hpi: 15.5±1.4; 24hpi: 5.5±3.3) and later at 72hpi (72.2±40.4). The maximum of expression of the JA-Ile receptor gene *COI1* was observed at 48hpi (2.8±1.2) which was coincident with the strong increase in expression level of the responsive–related gene *PR10* from 48hpi onwards (48hpi: 21.5±11.3; 72hpi:45.2±10.1).

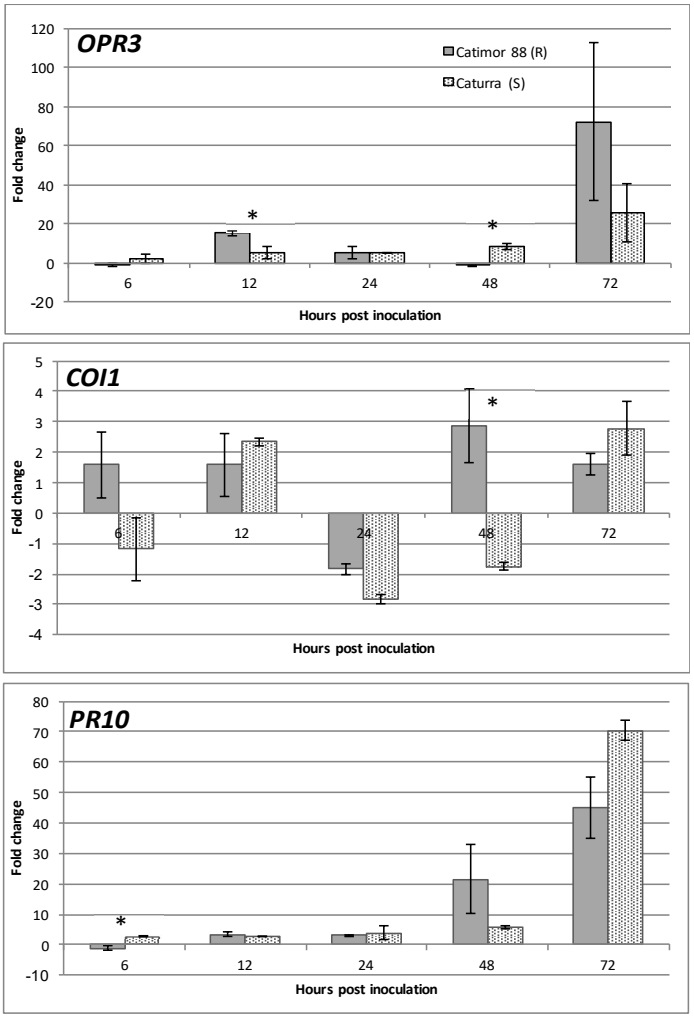

Figure 2.5 - qPCR expression analysis of JA pathway-related genes. Relative expression patterns of *OPR3* (biosynthesis), *COI1* (receptor) and *PR10* (responsive gene) obtained in Catimor 88 (R-resistant) and Caturra (S-susceptible) coffee varieties. Mean and standard deviation of three biological replicates is presented. Fold change as relative expression of gene expression between inoculated and control samples for each of the coffee varieties/inoculation time points. Asterisks (*) represent statistical significance ($p \leq 0.05$) of gene expression between the two coffee varieties and was determined by the non-parametric Mann–Whitney *U* test using IBM®SPSS® Statistics version 20.0 (SPSS Inc.) software.

An activation of the ET pathway was also observed in both varieties but with differences in the ET-receptor genes expression profiles (Fig. 2.6). In the susceptible variety, the biosynthesis-related genes *ACS5* and *ACO2* presented similar expression profiles being both up-regulated at 12hpi (*ACS5*: 4.1±0.2; *ACO2*: 1.8±1.3) and 72hpi (*ACS5*: 12±0.3; *ACO2*: 1.6±0.5) and down-regulated at 24hpi and 48hpi. ET-receptor gene *ETR1* and central ET signaling regulator *EIN2*, together with the negative ET pathway regulator *CTR1* showed similar expression

profiles being all up-regulated at 12hpi, and down-regulated at 24hpi followed by an up-regulation at 72hpi. ET-responsive gene *ERF1* was moderately activated from 6hpi to 48hpi, reaching a maximum value of expression at 72hpi (55.8±25.5). In the resistant variety, the biosynthesis-related gene *ACS5* was up-regulated at 12-24hpi (12hpi; 1.5±1.1; 24hpi: 1.8±1.3) while *ETR1*, *EIN2* and *CTR1* were down or non-regulated along the infection process. ET-responsive gene *ERF1* was moderately activated at all time points, with an increase in expression level at 48-72hpi (48hpi: 10.7±1.0; 72hpi: 8.5±1.6).

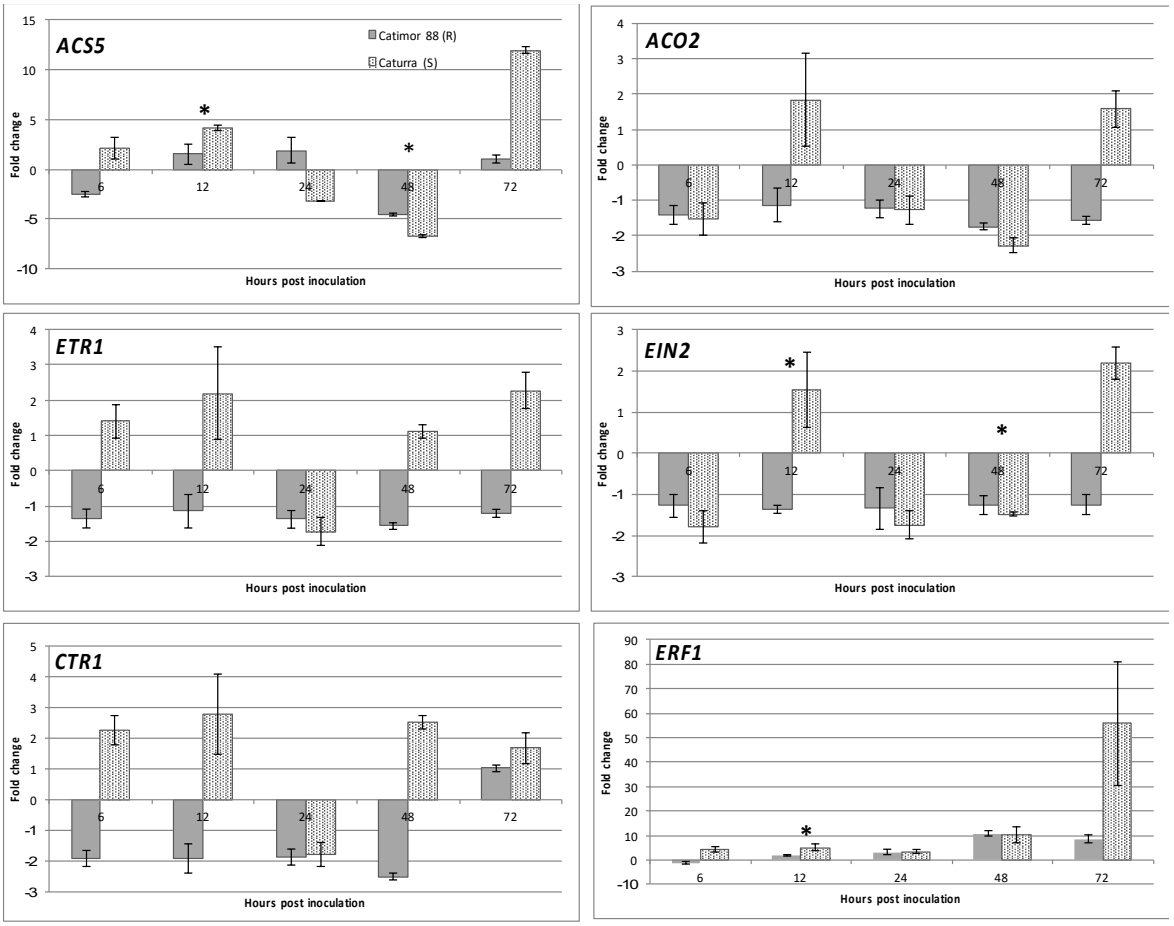

Figure 2.6 - qPCR expression analysis of ET pathway related genes. Relative expression patterns of *ACS5/ACSO2* (biosynthesis), *ETR1/EIN2* (receptors), *CTR1* (negative regulator) and *ERF1* (responsive gene) were obtained in Catimor 88 (R- resistant) and Caturra (S-susceptible) coffee varieties. Mean and standard deviation of three biological replicates is presented. Fold change as relative expression of gene expression between inoculated and control samples for each of the coffee varieties/inoculation time points. Asterisks (*) represent statistical significance ($p \leq 0.05$) of gene expression between the two coffee varieties and was determined by the non-parametric Mann–Whitney *U* test using IBM®SPSS® Statistics version 20.0 (SPSS Inc.) software.

2.4. Discussion

In this work, the involvement of phytohormones in the deployment of coffee susceptible and resistance response to *C. kahawae* was studied. In the two coffee varieties, no differences were observed in fungal development from conidia germination to differentiation of melanized appressoria and fungal penetration. In the majority of the infection sites of the susceptible variety, the fungus pursued its growth without apparent inhibition, first establishing a biotrophic interaction with the host cells and later (at 72pai) switching to a destructive necrotrophic phase, as described for many species of *Colletotrichum* (Torregrosa *et al.,* 2004; Munch *et al.,* 2008; Damm *et al.,* 2014; Kubo *et al.,* 2016). Conversely, as previously described by Silva *et al.,* (2006) and Loureiro *et al.,* (2012), in the resistant variety the restricted hyphal growth was associated with the hypersensitive-like host cell death (HR), and early accumulation of phenolic compounds both in cell walls and in the cytoplasmic contents. These responses were also observed in the susceptible variety, but in a significantly lower percentage of infection sites and did not prevent the fungal growth, as indicated by the appearance of typical anthracnose symptoms and the presence of acervuli. Time-course experiments carried out by Vargas *et al.,* (2012) also revealed that during the biotrophic growth in susceptible maize leaves, the hemibiotrophic fungus *C. graminicola* induced classical plant defence responses, such as the accumulation of reactive oxygen species and phenolic compounds. They hypothesized that it is the switch to necrotrophy that enables the fungus to evade the plant immune system and allows pathogenicity.

The classic view on the involvement of phytohormones in plant-pathogen interactions suggests that i) biotrophy is controlled by SA, while necrotrophy/hemibiotrophy is controlled by JA/ET, and that ii) SA is an antagonist of JA /ET (Glazebrook, 2005). Recent studies challenge these concepts revealing a very complex phytohormone self-regulation and cross-talk (Biles *et al.*, 1990; Shibata *et al.,* 2010; Sašek *et al.,* 2012; Duan *et al.,* 2014b; Figueiredo *et al.,* 2015; Amil-Ruiz *et al.,* 2016; Guerreiro *et al.,* 2016). Indeed, in some reports on plant-hemibiotrophic pathogen interactions, SA signaling seems to be relevant for the outcome of resistance (Liu *et al.,* 2007; Grellet-Bournonville *et al.,* 2012; Balmer *et al.,* 2013; Duan *et al.,* 2014a; Wanigasekara *et al.,* 2014; He *et al.,* 2016) while in others the role of SA is less clear (Prathima *et al.,* 2013; Litholdo *et al.,* 2015). In the coffee-*C. kahawae* interactions studied, the low expression of SA biosynthesis and receptor-related genes (*ICS2*/*PAL* and *NPR1*) turns also unclear the role of this phytohormone, although in coffee resistance against the biotrophic fungus *Hemileia vastatrix* a SA-dependent pathway seems to be involved (Diniz *et al.,* 2012; Sá *et al.,* 2014). The hormone-responsive gene *PR1* (commonly used as a SA pathway-marker)

was unexpectedly induced in both susceptible and resistant coffee varieties when challenged with *C. kahawae*, but in a greater magnitude in the resistant variety. The apparent lack of coordination between the *PR1* expression profile and the other selected SA-pathway markers under study (*ICS2*, *PAL* and *NPR1*) suggests that in this pathosystem, *PR1* induction could be mediated by other phytohormones than SA. In accordance, the knockout of *OsPAL06I* in rice challenged with *Magnaporthe oryzae* did not influence the expression of *PR1a* compared to wild-type, although the SA and JA levels were significantly reduced in roots (Duan *et al.,* 2014a). Tang *et al.,* (2010) reported a differential expression of *PR1* and chitinase genes in bananas matured and treated with ethephon (analog to ethylene), demonstrating that benzothiadizole (BTH – analog to SA) failed to induce them. Furthermore, an up-regulation of *PR1*, among others PRs, was observed after MeJA (methyl jasmonate) treatment (Duan *et al.,* 2014b; Amil-Ruiz *et al.,* 2016). The same authors related the increase of PRs expression with the increase of disease resistance, and identified a high degree of consistency between the concentration fluctuation of endogenous JAs and the expression of PRs, therefore suggesting that accumulation of JAs may be one inner factor for regulation of PRs.

Our results also revealed an induction of the JA pathway in both varieties, however it occurred earlier and/or in a greater magnitude in the resistant than in the susceptible variety. The biosynthesis-related gene *OPR3* was induced in both varieties during the appressorial melanization (12-24hpi) and the beginning of host cell responses (24hpi). Furthermore, an increase of the expression of JA-responsive gene *PR10* was observed at the beginning of fungal penetration (48pai) in the resistant variety, while in the susceptible variety it was coincident with the switch to necrotrophic fungal growth (72hpi). In a similar way, Ding *et al.,* (2011) observed that, when wheat was challenged with the hemibiotrophic fungus *Fusarium graminearum*, only the variety with a high level of resistance, when compared with a susceptible mutant, had an increase in *OPR3* activation, concluding that the induction of JA pathway was involved in plant defence. Figueiredo *et al.,* (2015) also observed a significant increase of *OPR3* and *COI1* expression in the resistant grapevine (*Vitis vinifera*) cultivar "Regent" at the first hours post inoculation with the biotrophic fungus *Plasmopora viticola,* when compared with the susceptible cultivar "Trincadeira", concluding that the timing of JA pathway induction was responsible for a set of efficient defence responses.

In our study, the earlier increase in *PR10* expression observed in the resistant variety as early as the fungal penetration stage, may reflect an early attempt of the coffee plant to halt the pathogen development, as is observed in other plant-pathogen interactions (Jaulneau *et al.,* 2010; Miles *et al.,* 2011; Amil-Ruiz *et al.,* 2016). Although the precise function of PR10

proteins is poorly understood, some exhibit antimicrobial activity, and DNase and/or RNase activity (Zubini *et al.,* 2009; Portal *et al.,* 2011). Recent studies also suggest that PR10 may have an important role in the control of phenylpropanoid and flavonoid biosynthesis and their transport to sites where they are needed, such as the reinforcement of the cell wall (Castro *et al.,* 2016). This seems to be in line with the accumulation of phenolic-like compounds in the host cell walls found in our study.

The ET pathway seems also to be induced in both coffee varieties however with a remarkable difference in the ET-receptor expression profile, which may reflect the complexity of the role and regulation of this hormone. The ET pathway induction may start at the earliest hours post inoculation, since at least one of the two ET biosynthesis enzymes were found to be up-regulated during the appressorial melanization (12-24hpi) and at the onset of host cell responses (24hpi) in both coffee varieties. It was also observed that at the switch to the nectrophic phase (72hpi), an up-regulation of both ET biosynthesis enzymes occurred but only in the susceptible variety. However, it is not clear which enzyme, ACO or ACS, is the rate-limiting factor in ET biosynthesis. Different reports have suggested that either ACO activity is the key step for controlling ethylene production (Ohtsubo *et al.,* 1999; Yu *et al.,* 2009) or ACS is the rate-limiting enzyme (Meng *et al.,* 2013). In susceptible *Nicotiana benthamiana* challenged with *C. orbiculare*, an induction of *ACO* gene was observed during the biotrophic phase, with a maximum expression coincident with the switch to necrotrophic phase after which expression started to decrease. However, in NbACO1-silenced plants inoculated with *C. orbiculare*, a higher number of leaf lesions appeared earlier when compared with control plants, suggesting that ET might have some role in plant defence although it was not sufficient to stop the disease progress in this interaction (Shan *et al.,* 2006). High induction of *ACS* and *ACO* genes was also reported in susceptible citrus flowers inoculated with *C. acutatum* with a continued increase of expression of both genes up to 7 days after inoculation (Li *et al.,* 2003; Lahey *et al.,* 2004). In this case, it was suggested that the induction of ET biosynthesis, and consequently the activation of ET pathway in susceptibility, was related with senescence of the tissues and promotion of young fruits drops, a characteristic of the Postbloom Fruit Drop disease (Li *et al.,* 2003; Lahey *et al.,* 2004). The regulation of ET-receptors plays a major role in the ET signaling pathway. Ethylene induces receptor degradation through the 26S proteasome at the same time that transcriptional activation of new receptors is promoted. By this way, newly synthesized receptors not yet bonded with ethylene will allow a downstream pathway inhibition as soon as the levels of the hormone decreases (Merchante *et al.,* 2013). In the susceptible coffee variety, the up/down-regulation profile of *ETR1* suggests that also in this

pathosystem, ET-receptors may undergo a similar regulation process. However, in the resistant variety, the repression of *ETR1* throughout the infection process may suggest a different receptor regulation or even an alternative role for ET. In fact, the ET receptors are largely redundant in the control of ethylene responses but some functional specificity among their different isoforms has recently been uncovered (Kevany *et al.,* 2008), together with evidences of a degree of complexity of ET receptors interaction with one another (Gallie, 2015). Therefore, either other ET receptors or isoforms are activated in response to pathogen attack and/or ET pathway activation is related with other functions rather than pathogen defence only. *ERF1* gene is a transcriptional factor of ET induced defence genes while it is an ET-responsive gene itself, being commonly used as an ET-pathway marker (Shan *et al.,* 2006; Sugano *et al.,* 2013). The *ERF1* gene was moderately induced in the resistant coffee variety contrasting with the increasing up-regulation in the susceptible variety, which culminates in a strong activation at the beginning of the necrotrophic phase. A comprehensive analysis of the ethylene role in plant response to pathogen attack was undertaken by Chen *et al.,* (2003). When challenged with *C. destructivum*, the susceptible variety of *N. tabacum* produced ET in two distinct moments, the first during the biotrophic phase and the second during the necrotrophic phase (when leaf tissue was severely damaged or dead). It was then suggested that the first peak of ET production was related with the onset of unsuccessful defence responses and the second peak with the senescence of infected tissues. Although no ET production was measured in our assay, our results suggest that ET pathway activation in the susceptible variety may be related with tissue damage promoted by the fungal necrotrophic phase. In fact, it has been suggested that in later infection stages, hemibiotrophic pathogens may produce ethylene and deliver effectors or phytotoxins that manipulate the plant to produce ethylene for entering the necrotrophic stage of infection (Groen *et al.,* 2014 and references therein), and thus overcome plant defences.

Overall, our results suggest that in both coffee varieties, the immune system enable the perception of the pathogen attack as the infection process starts, being the expression of resistance and susceptibility conditioned by the magnitude and/or timing of defence responses. In the resistant variety, the earlier and strong induction of JA biosynthesis and receptor-related genes, together with the activation of PR genes (*PR1* and *PR10*) seems to be important players in the set of defences that resulted in the arrest of fungal growth. The stronger activation of the ethylene responsive-related gene *ERF1* at the switch to the necrotrophic fungal growth, that is coordinated with the activation profile of biosynthesis and receptor-related genes, suggests that this hormone may be relevant in susceptibility, although its possible involvement in defence

responses is not discarded. To the best of our knowledge, this work represents the first attempt to unveil the involvement of phytohormones pathways in coffee-*C. kahawae* interaction making possible to engage on new exploratory functional assays. Future studies focusing on RNA-Seq analysis will provide a better understanding of the molecular mechanisms underlying the defence responses in this pathosystem.

2.5. References

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Chapter 3

Fungal penetration associated with recognition, signaling and defence-related genes and peroxidase activity during the resistance response of coffee to *Colletotrichum kahawae*

The data presented in this chapter is in preparation to be submitted in Physiological and Molecular Plant Pathology

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3. Fungal penetration associated with recognition, signaling and defence-related genes and peroxidase activity during the resistance response of coffee to *Colletotrichum kahawae*

Abstract

Resistant and susceptible coffee varieties were used aiming to unveil genes and proteins putatively involved in the resistance response to *Colletotrichum kahawae*. Gene expression analysis revealed a stronger induction of recognition, signaling and cell wall modification genes in the resistant variety during fungal penetration. It was also observed the increase of total peroxidase activity and the induction of *lignin-forming anionic peroxidase-like* gene which was further supported by the increase of an anionic isoenzyme. Peroxidases localized in the walls of host cells at infection sites can be related with cell wall lignification. Altogether, these defence responses contribute to restricting fungal growth.

3.1. Introduction

Plants have employed a multi-layered system of defences to protect themselves from various pathogens. In addition to passive mechanisms plants have evolved at least two lines of active defences (Jones & Dangl, 2006; Hückelhoven, 2007; Bonardi & Dangl, 2012; Miedes *et al.,* 2014). The first line provides basal defence against all potential pathogens and is based on the recognition of conserved pathogen-associated molecular patterns (PAMPs), by so-called pattern recognition receptors (PRRs) that activate PAMP-triggered immunity (PTI). The second layer of defence is activated when a given pathogen-derived molecule, called effector, is 'specifically recognized' by plant receptor proteins encoded by R genes, resulting in effector-triggered immunity (ETI). Once activated, both PTI and ETI induce a downstream of similar defences, such as rapid accumulation of reactive oxygen species (ROS), changes in cellular ion fluxes, activation of protein kinase cascades, production of stress-related hormones, cell wall modifications and changes in protein and gene expression (Jones & Dangl, 2006; De Wit *et al.,* 2009). ETI, when compared with PTI, is associated with more sustained and robust immune responses including hypersensitive reaction (HR), a form of programmed plant cell death localized at the infection sites (Jones & Dangl, 2006) which is considered to be one of the most important factors in the restriction of the pathogen growth, particularly of obligate biotrophs (Heath, 2000; Lam *et al.*, 2001). HR is often associated with the production of ROS, increased

activity of oxidative enzymes, loss of cell membrane integrity and activation of defence-related genes (pathogenesis-related or PR genes) (Heath, 2000).

The hemibiotrophic fungus *Colletotrichum kahawae* J. M. Waller & P.D. Bridge is the causal agent of Coffee Berry Disease (CBD). This disease is considered the biggest threat to Arabica coffee production in Africa leading to up to 50-80% of crop losses, in years of severe epidemics if no control measures are applied. CBD is so far restricted to Africa, but the risk of its introduction in America and Asia represents a serious concern (Silva *et al.*, 2006; van der Vossen & Walyaro, 2009). *C. kahawae* infects several coffee organs, but major losses result from the infection of green berries during their expanding stage with visible symptoms of dark, sunken anthracnose-like lesions on the green pulp (Hindorf & Omondi, 2011). Coffee breeding for CBD resistance proved to be a successful method of minimizing yield losses and controlling the disease.

Coffee resistance/immunity to *C. kahawae* is controlled by major genes in different loci (van der Vossen & Walyaro, 1980; Omondi & Pinard, 2006; Gichuru *et al.*, 2008). At cytological level, resistance is characterized by restricted fungal growth associated with: (i) cell wall modifications, such as accumulation of phenolic-like compounds followed by lignification, thickening and deposition of suberin; (ii) deployment of HR identified by several ultrastructural changes, including membrane breakdown, changes in the appearance of chloroplasts and the nucleus and coagulation of the cytoplasm; (iii) early deposition of callose around intracellular hyphae (Masaba & van der Vossen, 1982; Silva *et al.*, 2006; Loureiro *et al.*, 2012; Diniz *et al.*, 2017). Furthermore, the expression analysis of phytohormone-related genes revealed a stronger activation of jasmonic acid-dependent pathway during coffee resistance to *C. kahawae* (Diniz *et al.*, 2017).

Despite all the studies performed so far, relatively little research progress has been made in gaining new insights into the biochemical and molecular basis of coffee immunity/resistance to *C. kahawae.* In this work, the coffee variety Catimor 88 was used aiming to unveil the candidate genes and proteins putatively involved in the resistance response to *C. kahawae* (isolate Que 2 from Kenya), in a comparative analysis with the susceptible variety Caturra. Quantitative real-time PCR (qPCR) was used to evaluate the expression of genes related to pathogen recognition, signaling, cell wall modifications and peroxidases (related to ROS generation and lignification). Further analysis of oxidases by spectrophotometry, electrophoresis, and histochemistry were also performed.

3.2. Materials and methods

3.2.1. Biological material and inoculation

Coffee hypocotyls were used in experimental assays, given the reported existence of a correlation (r = 0.73 – 0.80) between the pre-selection test on hypocotyls and mature plant resistance in the field (van der Vossen *et al.*, 1976). Catimor 88 (Timor hybrid derivative, used with success as a source of resistance to *C. kahawae* in Kenyan breeding programs) and Caturra (CIFC 19/1 – *Coffea arabica* L.) were used as resistant and susceptible coffee varieties to *C. kahawae* isolate Que2 (from Kenya), respectively. Coffee seeds were sown and grown according to the conditions previously described (Diniz *et al.*, 2017).

Conidia of *C. kahawae* isolate Que2, retrieved from a *C. kahawae* CIFC/ISA collection, were obtained from a 7-day-old culture grown on malt extract agar (MEA) at 22°C (Rodrigues *et al.*, 1991). Hypocotyls of both coffee varieties were inoculated according to the technique described by van der Vossen *et al.*, (1976) with slight modifications (Diniz *et al.*, 2017). Control hypocotyls were sprayed with water only. Inoculated and control hypocotyls were maintained under the same conditions (Diniz *et al.*, 2017).

3.2.2. Sample collection

The time points selected in this work to collect the inoculated hypocotyls were based on previous microscopic analysis (Diniz *et al.*, 2017), and corresponded to the following stages of the infection: (i) **12 hours post inoculation (hpi)**- Conidia germination and about 50% of melanized appressoria (in both coffee varieties); ii) **24hpi** – close to 100% of melanized appressoria (both varieties) and beginning of host responses [cell wall modifications (accumulation of phenolic-like compounds) and HR], observed in a significantly higher percentage of infection sites in the resistant (6%) than in the susceptible variety (2%); (iii) **48hpi** - fungal penetration, biotrophic growth and higher progression of the host responses in the resistant variety (26% of infection sites and only 4% in the susceptible); iv) **72hpi** - switch to necrotrophic phase (susceptible variety) in opposition to restricted fungal growth associated with host responses in more than 50% of infection sites (resistant variety).

The inoculated and control hypocotyls collected at each time point were immediately frozen in liquid nitrogen and stored at -80°C until used.

3.2.3. RNA extraction and cDNA synthesis

Infected and control hypocotyls of both varieties were harvested at 12, 24, 48 and 72hpi. Procedures for RNA extraction, purification and synthesis of complementary DNA (cDNA) were performed as described previously (Diniz *et al.*, 2017).

3.2.4. Primer design

The nine genes selected to be studied by qPCR, were chosen based in their biological function, i.e. pathogen recognition and signaling (Receptor-like kinase – *RLK,* Leucine rich repeat receptor-like serine/threonine-protein kinase At2g16250 – *LRR-K*, Proline-rich receptor-like protein kinase – *PERK3*, Calmodulin-like protein – *CML,* Patatin-like phospholipase - *PTL*), cell wall modifications (UDP-arabinose 4-epimerase 1- *MUR4*, Pectinesterase/pectinesterase inhibitor 41- *PME41*) and peroxidases (Cationic peroxidase 2 – *PNC2*, Lignin-forming anionic peroxidase-like – *PER4*). These genes were retrieved from a coffee RNA-seq database (Fino *et al.*, 2015) as being orthologous to previously described genes in *Arabidopsis thaliana* (TAIR database:www.arabidopsis.org). Tubulin beta-9 (*β-Tub9*)/ribosomal protein S24 (*S24)* and Insulin Degrading Enzyme (*IDE*)/*S24* were used as reference genes for susceptible and resistant varieties samples, respectively (Figueiredo *et al.,* 2013). Coffee specific primers (Table 3.1) were designed with PrimerSelect version 5.0 (DNAStar, Inc.) using the following parameters: amplicon length 70 and 200 bp; size between 17 and 24bp; annealing temperature (Ta) between 55°C and 60°C and GC content± 50%.

Table 3. 1. Primer sequences for qPCR analysis for target and reference genes

Genes	Coffee source name	Name	Primer Sequence (5'-3')	Primer lenght (bp)	Amplicon lenght (bp)	Ta (ºc)	Tm (ºc)	PCR efficiency
RLK	CF589181	Receptor-like kinase	Fw: ATGGGAGAAAAGAATGGCAGAAG Rev: GGCCAATTACAGTTTGAAAACACC	23 24	189	55	81.2	100
LRR-K	Scaffold7309	Putative LRR receptor-like serine/ threonine-protein kinase At2g16250	Fw: CATCATCAATACCATCACCAC Rev: GGAATTCGGGTAGTGGTG	21 18	185	60	83	91
PERK3	Scaffold151816	Proline-rich receptor-like protein kinase	Fw: CCCTTAGTATCAGTAGTAGAGCCT Rev: TCTCCTAAAGTTGATGTCTATGCC	24 24	101	55	78	92
CML	C255095	Calmodulin-binding protein-like	Fw: GCATGTTTCAACGTCACCTC Rev: CCATCTTTTCCAATCTTTTCC	20 21	165	58	81.5	89
PTL	Scaffold10451	Patatin-like phospholipase	Fw: CACACTTAATGCTGGGACAA Rev: GTTGTCATCCCTACTTTCGA	20 20	151	58	81.5	92
MUR4	Scaffold19499	UDP-arabinose 4-epimerase 1-like	Fw: GGATATCTGGTGCTTGTTTTG Rev: GCCATCTGAAGTTTTATAGTCT	21 22	86	60	76.7	99
PME41	C292479	Putative Probable pectinesterase/ pectinesterase inhibitor 41	Fw: GGTAACCTTCAAAACTACAC Rev: CGGCCGGAGCAATAAAAC	20 18	87	58	77.1	92
PNC2	Scaffold 63431	Cationic peroxidase 2	Fw: CGACGGTTCTATCCTCATTGAC Rev: GTGATTCTGTTGTGCTGAGT	22 21	178	60	84	93
PER4	Scaffold 98714	Lignin-forming anionic peroxidase-like	Fw: CTACTTGCCCTAATGCTCTCAC Rev: AGGGTTGTGACGGATCAGTA	22 20	134	60	83	95
IDE a	Isotig10635	Insuline Degrading Enzyme	Fw: TGATCTAAGCTGGTGGAAAGC Rev: TCAGGTGCATCAGGATGATT	21 20	91	55	76.5	99
S24 a	SNG-U349723 *	Ribosomal protein S24	Fw: GCCCAAATATCGGCTTATCA Rev: TCTTCTTGGCCCTGTTCTTC	20 20	92	60	76	95
β-Tub9 a	Isotig08544	Tubulin beta-9	Fw: ACCCTCCAGCAAACTGATGA Rev: AGGATGCCACTGCTGATGAT	20 20	100	55	77.3	92

bp – base pairs, Ta – Annealing temperature, Tm – Melting temperature

* Unigene accession number according to the SOL Genomics Network.

[a] Figueiredo *et al.*, 2013.

3.2.5. Quantitative real-time PCR

The qPCR experiments were carried out using SYBR Green Supermix (Bio-Rad) in an iQ5 real-time thermal cycler (Bio-Rad) as previously described (Diniz *et al.,* 2017). Each set of reactions included a negative control with no template. For each gene of interest, the amplification efficiency was determined using the LinRegPCR version 2013.0. Dissociation curves (Fig. S3.1) and agarose gel electrophoresis were used to analyse non-specific PCR products. Three biological replicates and two technical replicates were used for each sample. Relative gene expression (fold change) was calculated according to Hellemans *et al.,* (2007).

3.2.6. Preparation of protein extracts for enzymatic assays

Protein extracts were prepared from control and inoculated hypocotyls of resistant and susceptible coffee varieties collected at 12, 24, 48 and 72hpi.

For total peroxidase (POD) and polyphenol oxidase (PPO) activities (spectrophotometric assay) 300mg of hypocotyls were grounded to powder with liquid nitrogen and homogenised in ice-cold 50mM sodium acetate buffer pH 5.6, containing 10% PVPP (w/v). The suspensions were stirred for 30 min at 4ºC and centrifuged at 27 000*g* for 15 min at 4ºC (Avantis 26XPI,

Beckman Coulter). The pellets were discarded and the enzymatic extract was stored at -20ºC until used.

For isoenzymatic POD and PPO activities in gels 300mg of hypocotyls were grounded to powder with liquid nitrogen and homogenised in ice-cold 0.2M Tris-HCl buffer pH 8.2, 0.14M NaCl, 2% PVP K25, 0.05% Tween 20, 0.2% BSA, 2% sodium sulfite and PVPP [1:1, w/w]. The suspensions were centrifuged at 25 000*g* for 15 min at 4ºC (microfuge 22R, Beckman Coulter). This fraction was subsequently concentrated in centrifugal Vivaspin500 (Sartorius) and stored at -20ºC until used.

The protein content of the extracts was quantified based on a modified Bradford method using the Bio-Rad protein assay for microtiter plates (Asys UVM 340, Biochrom). A bovine serum albumin (BSA) was used as standard and absorbance was measured at 595nm.

3.2.7. Total enzymatic activities

Total POD and PPO activities were determined according to the method of Cavalcanti *et al.,* (2006) modified for microtiter plates (Asys UVM 340, Biochrom). Duplicate samples of tissue were taken for enzyme extraction at different times points post inoculations. Experiments were repeated at least three times.

POD activity was determined by adding 40µl of the crude enzymatic extract to 160µl of 50mM sodium acetate buffer pH 5.2, containing 50mM guaiacol and 60mM hydrogen peroxide (H_2O_2). Guaiacol was used as the hydrogen donor and the H_2O_2 as the enzyme substrate. The absorbance was measured at 480nm and expressed as ΔD.O. min/mg protein.

PPO activity was determined by adding 40µl of the crude enzymatic extract to 160µl of 50mM sodium phosphate buffer pH 7.0, containing 100mM catechol. The absorbance was measured at 410 nm and expressed as ΔD.O. min/mg protein.

3.2.8. Electrophoretic methods

Polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (1970) and carried out in a vertical slab 0.75mm thick (Mini-Protean Tetra cell, Bio-Rad) with a 4% stacking gel (pH 6.8) and a 7.5% resolution gel (pH 8.8). Wells were load with 20µg of protein per sample or with 5µl of Pre-stained SDS-PAGE Low Range Standard (reference 161-0305,

Bio-Rad). Electrophoresis was performed at a constant current of 200V for 45 min at 4ºC using Tris-glycine pH 8.3 as running buffer.

Isoelectric focusing electrophoresis (IEF) was performed according to Robertson *et al.,* (1987) and carried out in a vertical slab 1.5mm thick (Mini-Protean Tetra cell, Bio-Rad) with 5% polyacrylamide and 5% ampholytes [2.5% Servalyt® 3–10 and 2.5% Servalyt® 5–6 from SERVA Electrophoresis GmbH]. Wells were load with 10µg of protein per sample or with 5µl of IEF Standard Broad Range pI 4.45-9.6 (reference 161-0310, Bio-Rad). Electrophoresis was performed at 4ºC for 1.5 hours: 45 min at a constant voltage of 200V followed by a second period of 45 min at a constant voltage of 400V using 25mM NaOH solution in the cathode and 20mM acetic acid solution in the anode.

3.2.9. Isoenzymatic activities in gel

POD activity in the gel was determined according to the method described by Silva *et al.*, (2008) based on Smith & Hammerschmidt (1988). Briefly, after running, gels were incubated in 50mM citrate phosphate buffer pH 6.0 containing 0.2% guaiacol, 0.2% H_2O_2 and 1% 12mM 3-amino-9-ethylcarbazole in dimethylformamide. Brown/red bands were visualized within 10 min.

PPO activity in gel was evaluated according to the method described by Cantos *et al.*, (2002) with slight modifications. Briefly, after running, gels were incubated in 0.1M sodium acetate buffer pH 5.0 containing 10mM catechol, 5mM 3-methyl-2-benzothiazolinonehydrazone (MBTH) and10µg/ml catalase. Deep blue bands were visualized within 5-10 min.

The image of each stained gel was captured (ImageScanner, Amersham Biosciences) and analysed by CLIQ 1D PRO (TotalLab).

3.2.10. Histochemical localization of POD

Localization of POD in cross sections of inoculated hypocotyls (24, 48 and 72hpi) was performed according to the guaiacol test, as previously described (Maehly & Chance, 1954; Silva *et al.,* 2008). The presence of POD was indicated by the accumulation of tetraguaiacol as a brown-red coloration.

3.2.11. Statistical analysis

Statistical significance ($p \leq 0.05$) of gene expression between the two coffee varieties was determined by the non-parametric Mann–Whitney *U* test. Data concerning POD and PPO activities were presented as the combined values of two experiments, and Student *t*-test for statistical analysis was used. All the statistic tests were performed in IBM®SPSS® Statistics version 20.0 (SPSS Inc.).

3.3. Results

3.3.1. Relative gene expression

The expression of genes related to pathogen recognition, signaling, cell wall modifications and peroxidases was analysed by qPCR (Fig. 3.1).

The recognition, signaling and cell wall modifications genes (*RLK*, *LRR-K*, *PERK3*, *CML*, *PTL, MUR4* and *PME41*) were up-regulated at all time points of the infection process in both varieties.

In the resistant variety, comparatively to the susceptible one, a significant increase in expression of the recognition and signaling genes was obtained at 48hpi (, *RLK:* 40.6±19.8, *LRR-K*:7.5±0.4, *PERK3*: 95.3±6.3, *CML*: 22.6±7.5 and *PTL:*88.8±1.5).

In the susceptible, all these genes reached a maximum of expression at 72hpi (*RLK*: 33.7±2.1; *LRR-K*: 8.7±0.0; *PREK3*: 64.4±1.2, *CML*: 12.4±4.9; *PTL*: 138.8±13.8), but only the values of *LRR-K* and *PERK3* were significantly different from the resistant variety. The cell wall modifications genes (*MUR4* and *PME41*) reached a maximum of expression at 72hpi in both varieties but with higher values in the resistant (resistant – *MUR4*: 242.9±6.6, *PME41*: 504.7±6.0; susceptible – *MUR4*: 184.9±51.6, *PME41*: 58.1±27.8). However, at 48hpi a significantly increase in the expression of *PME41* gene was obtained in the resistant variety while at this time in the susceptible the increase in expression was obtained for *MUR4* gene.

The peroxidase genes, *PNC2* and *PER4*, were up-regulated at all time points of the infection process, except for *PNC2* at 12hpi. In both varieties, a maximum of gene expression was observed at 48hpi for *PNC2* (resistant: 2.5±0.8; susceptible: 2.5±1.3) and at 72hpi for *PER4* (resistant: 7.4±1.4; susceptible: 3.9±2.6). Significant differences in these genes expression was only found in *PER4* at 12hpi, being more expressed in the resistant variety when compared with the susceptible one.

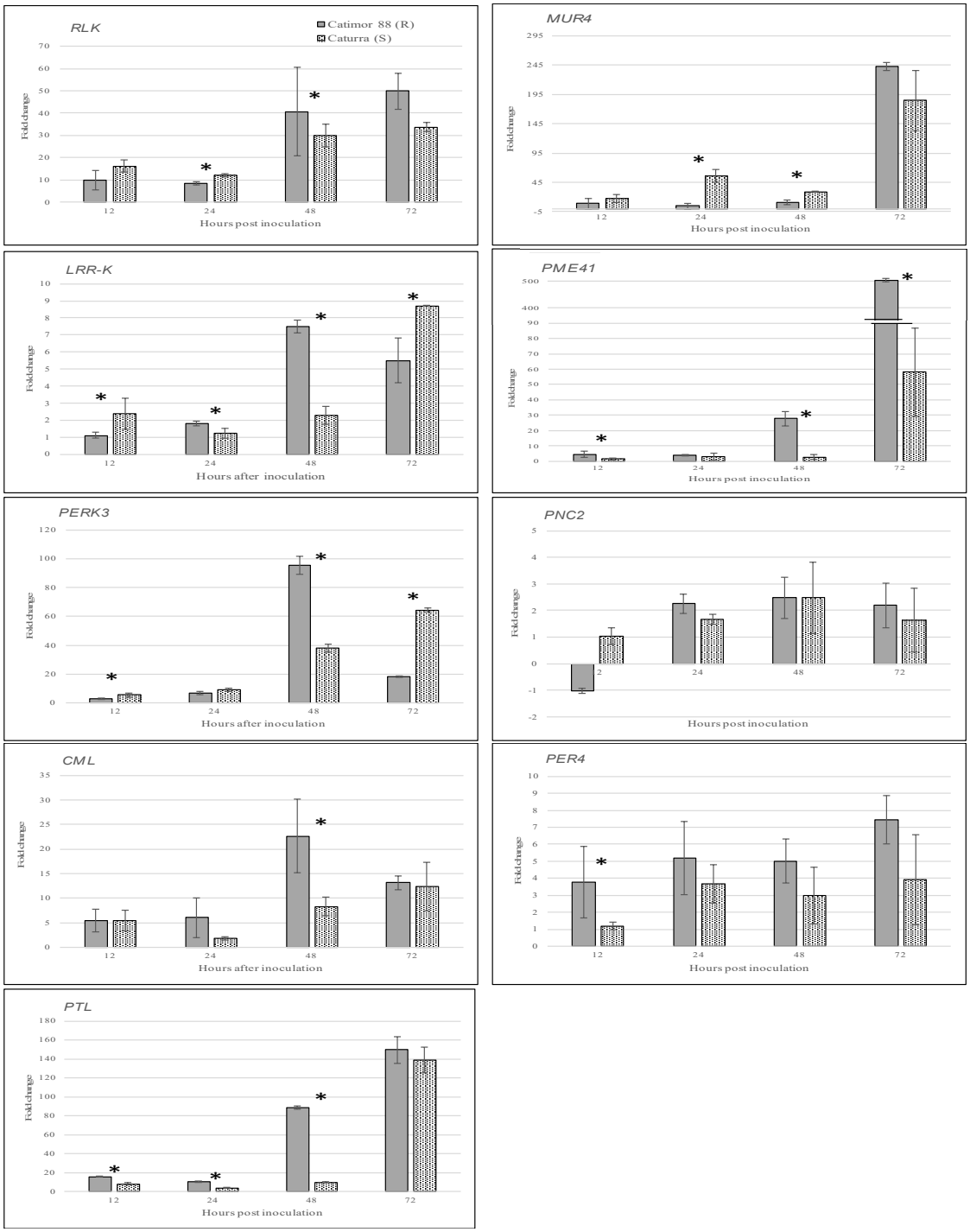

Figure 3. 1- qPCR expression analysis of defence-related genes. Relative expression patterns of pathogen recognition and signaling (*RLK*, *LRR-K, PERK3*, *CML, PTL*), cell wall modifications (*MUR1, PME41*) and peroxidases (*PNC2, PER4*) were obtained in non-inoculated and inoculated hypocotyls of Catimor 88 (R - resistant) and Caturra (S - susceptible) varieties. Hypocotyls were collected at different times post inoculation with *Colletotrichum kahawae* (12–72 h). Fold change as relative expression of gene expression between inoculated and control samples for each of the coffee varieties/inoculation time-points. Error bars represent the standard deviation pf the mean from three independent biological experiments. Asterisks (*) represent statistical significance ($p \leq 0.05$) of gene expression between the two coffee varieties was determined by the non-parametric Mann–Whitney *U* test using IBM®SPSS® Statistics version 20.0 (SPSS Inc., USA) software.

3.3.2. Total POD and PPO activities

In the resistant variety, significant differences in total POD activity were detected between inoculated and control hypocotyls by 48hpi onwards, while for total PPO activity significant differences were detected at 72hpi (Fig. 3.2A and C). On the contrary in the susceptible variety no differences in total POD and PPO activities were detected between inoculated and control samples (Fig. 3.2B and D).

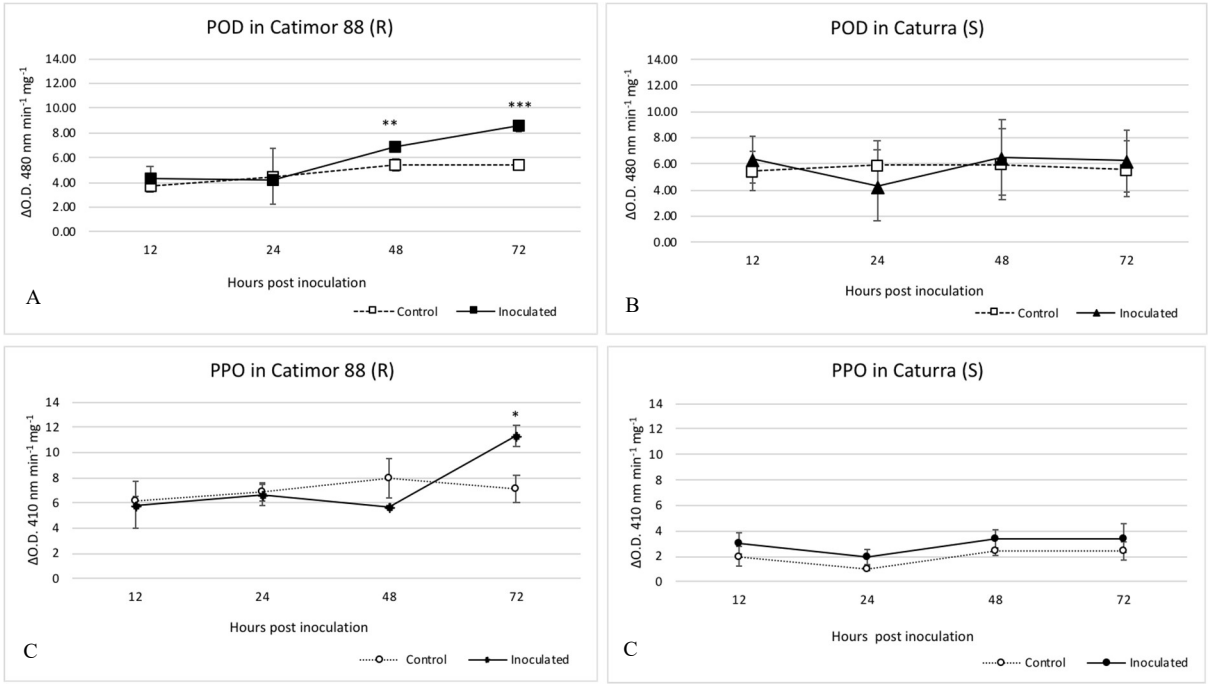

Figure 3. 2 – POD and PPO activities in control (non-inoculated) and inoculated hypocotyls of Catimor 88 (R - resistant) and Caturra (S - susceptible) varieties. Total POD and PPO activities were expressed as Δ O.D. 480nm $min^{-1}mg^{-1}$ of protein and Δ O.D. 410 nm $min^{-1}mg^{-1}$ of protein, respectively. Hypocotyls were collected at different times post inoculation with *Colletotrichum kahawae* (12–72 h). In the resistant variety, total POD activity was significantly different from the control at 48 hpi (*t* test =4.53; P≤0.01) and 72 hpi (*t* test = 9.44; P≤0.001). In the resistant variety, total PPO activity was significantly different from the control at 72 hpi (*t*-test =3.03; p≤0.05).

3.3.3. POD and PPO isoenzymes

POD isoenzymatic profiles obtained in PAGE gels showed differences between both varieties (Fig. 3.3A and B). In the resistant variety, two POD isoforms (134 and 39kDa) were detected revealing an increase in activity at 48h and 72h after challenge with *C. kawahae* (Fig. 3.3A). In the susceptible variety, only one isoform (45kDa) was detected, but no differences in activity were observed between inoculated and control hypocotyls (Fig. 3.3B).

In IEF gels a similar isoenzymatic profile of POD, with three anionic isoforms, was obtained for both varieties (Fig. 3.3C and D). In the resistant variety, the isoenzymes detected (pI 6.3, 6.1 and 4.8) changed differentially along the infection process; particularly the isoform pI 6.1 which activity increased by 24-72hpi in the inoculated hypocotyls (Fig. 3.3C). In the susceptible variety, the activity of the isoenzymes pI 6.3, 5.8 and 4.8 also changed; the isoform pI 6.3 increased by 48-72hpi, but for the isoform pI 5.8 almost no activity was detected in the inoculated hypocotyls along the infection process (Fig. 3.3D).

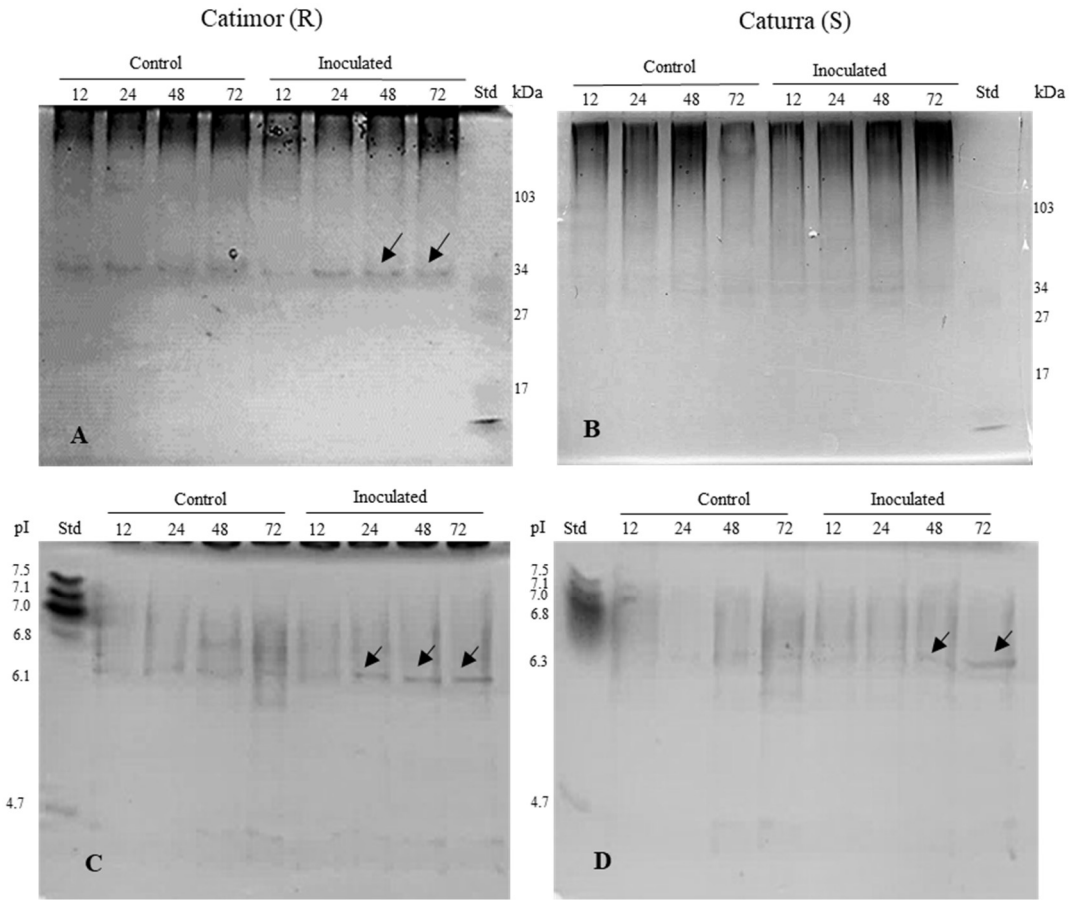

Figure 3. 3 - POD isoenzyme identification on polyacrylamide gels stained with guaiacol and 3-amino-9-ethylcarbazole. PAGE gels: A and B; IEF gels: C and D. POD was extracted from control (non-inoculated) and inoculated hypocotyls of Catimor 88 (R - resistant) and Caturra (S - susceptible) varieties. Hypocotyls were collected at different times post inoculation with *Colletotrichum kahawae* (12–72 h). Twenty micrograms of protein were loaded per lane in PAGE gels and Pre-stained PAGE Low range (reference 161-0305, Bio-Rad) was used as standard (Std). Ten micrograms of protein were loaded per lane in IEF gels and IEF Standard (reference 161-0310, Bio-rad) was used as standard (Std); pI – Isoelectric point.

In the PPO isoenzymatic profiles obtained in PAGE and IEF gels for both varieties, no differences were observed between inoculated and non-inoculated hypocotyls (Fig. S3.2A-D).

However, differences in the molecular weight and isoelectric points of the isoforms were obtained when both varieties were compared. In the resistant variety two isoforms with 139/54 kDa and two with pI 8.7/6.3 were detected (Fig. S3.2 A and C), while in the susceptible two isoforms with 133/45 kDa and two pI 8.3/6.3 were observed (Fig. S3.2B and D).

3.3.4. Histochemical localization of POD

The presence of POD, indicated by the brown-red coloration, began to be detected at 24hpi in the cell walls only or in both the walls and the cytoplasmic contents of epidermal cells at the infection sites, extending later to the adjacent cortex cells (Fig. 3.4A and B).

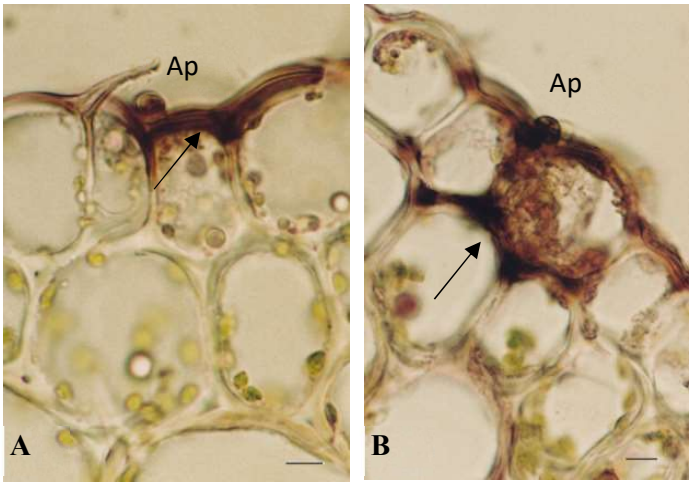

Figure 3. 4 - Histochemical localization of POD in hypocotyls of variety Catimor 88 (resistant) upon inoculation with *Colletotrichum kahawae.* Figs. 4A and 4B - Infection sites showing melanized appressoria (Ap) and POD in the walls of epidermal cells (arrow), 24 hpi (Fig. 4A) and in the walls and cytoplasmic content of an infected epidermal cell (arrow), 72 hpi (Fig. 4B). The presence of POD was indicated as a brown-red coloration, by guaiacol test under light microscope observations; bar = 10 μm.

3.4. Discussion

We investigated the genes and proteins associated with pathogen recognition, signaling, cell wall modifications and oxidases displayed during the resistance response of coffee to *C. kahawae*.

The expression pattern of pathogen recognition and signaling genes suggests that the resistant and susceptible varieties are able to perceive the pathogen presence at early stages of the infection process. However, in the resistant variety, a significant increase in gene expression

related to recognition and signaling was coincident with fungal penetration at 48hpi, while in the susceptible variety the maximum expression of these genes was observed later, at 72hpi, when the fungus switches to necrotrophy. The earlier induction of transmembrane-receptor genes during the resistance response of coffee against *Hemileia vastatrix* (Fernandez *et al.,* 2004; Diniz *et al.,* 2012), as well as in *Vitis flexuos*a challenged with *C. gloesporioides* (Islam *et al.,* 2015), has also been observed. Transmembrane-receptors have been considered an important element in pathogen recognition and in plant resistance response since they perceive external and internal signaling molecules, the first step in plant induction of defence responses. RLKs that includes proline-rich receptor-like protein kinase (PERKs) and leucine-rich repeat RLKs (LRR-RLKs) are usually composed of: a distinct extracellular domain that can specifically sense signals (e.g., phytohormones, proteins, small peptides, polysaccharides), a single transmembrane domain and an intracellular kinase domain. Once activated, these transmembrane-receptors often form complexes with other receptors resulting in distinct signaling pathways that transmit the signal into the cell for the necessary differential outputs like, development regulation, growth, and immunity (Burkart & Stahl, 2017).

Our results showed that perception of pathogen seems to be followed by calcium (*CML*) and lipid (*PTL*) signaling cascades, being both genes significantly more expressed in resistant than in the susceptible coffee variety, during fungal penetration and beginning of biotrophic growth (48hpi). A Ca^{2+} ion influx is one of the earliest events in challenged cells that relies on Ca^{2+} sensors, such as the calmodulin-like proteins, to set the signal by binding to other proteins resulting in activation or inactivation of inter-acting proteins which will culminate with the induction of defence-related genes such as pathogenesis-related proteins (PRs) and HR (Reddy *et al.,* 2011; Ranty *et al.,* 2016). PTLs are responsible for the non-specific hydrolysis of phospholipids and mediate the biosynthesis of oxylipins in plant innate immunity (La Camera *et al.,* 2009; Kim *et al.,* 2014) being closely related to the JA pathway (La Camera *et al.,* 2009; Bethke *et al.,* 2014; Kim *et al.,* 2014). A high degree of consistency between the resistance of coffee to *C. kahawae* with the increase in gene expression of PRs (*PR1* and *PR10*), induction of HR and earlier and stronger activation of JA pathway had been previously found (Diniz *et al.,* 2017).

MUR4 (UDP-arabinose 4-epimerase 1) and *PME41* (Pectinesterase/pectinesterase inhibitor 41) genes are related to cell wall modification. MUR4 catalyzes the 4-epimerization of UDP-D-xylose to UDP-L-arabinose, the nucleotide sugar used by glycosyltransferases in the arabinosylation of cell wall polysaccharides and wall-resident proteoglycans, which can lead to alterations in covalent-linking between different cell wall polymers (Burget *et al.,* 2003).

For instance, covalent ester-ester bridge between polysaccharides and lignins are formed by ferulic acid on arabinoxylans (Iivama *et al.,* 1990). On the other hand ferulic acids can be esterified to pectins via arabinose residues (Lamb *et al.,* 1990) which are the main component of pectin arabinan (Kotake *et al.,* 2016). PME proteins catalyse the demethyl-esterification of the homogalacturonan pectin domain, enabling cell wall remodelling (Lionetti *et al.,* 2012; Day *et al.,* 2013). The degree and pattern of demethyl-esterification affect the accumulation of pectin breakdown fragments like oligogalacturonides (OGs) that are released from plant cell walls, upon partial degradation of homogalacturonan, turning the cell wall more susceptible to the microbial degrading enzymes (Ferrari *et al.,* 2013). However once de-esterified, OGs can act as an elicitor of defence responses that are perceived by a wall associated kinase to reinforce the plant innate immunity (Lionetti *et al.,* 2012; Ferrari *et al.,* 2013). Furthermore, Bethke *et al.,* (2014) observed that PME activity was only reduced in *Arabidopsis* mutants *dde2* and *coi1* (both blocked in JA signaling) and it was indistinguishable between the wild type and the other mutants (*ein2* - blocked in ET signaling, *sid2* - blocked in SA signaling, *pad4* - PAD4 is required for SA signaling), concluding that pathogen-induced PME activity is also affected by JA signaling. Interestingly, we have observed an increase in *MUR4* and *PME41* gene expression in both varieties, suggesting that cell wall structure is modified as a response to pathogen presence. However, compared to the susceptible, in the resistance variety, a significant increase in *PME41* expression was detected at 48hpi and 72hpi, which was coincident with a higher progression of host responses and fungal growth restriction, previously observed by Diniz *et al.,* (2017). In *Arabidopsis,* the increase of PMEs activity resulted in a decreased pectin methyl-esterification of the cell wall when challenged with pathogens with distinct lifestyles [*Pseudomonas syringae* (hemibiotrophic bacteria) and *Alternaria brassicicola* (necrotrophic fungus)] (Bethke *et al.,* 2014). In line with this findings, an increase in the hydrolytic activity of PME proteins associated with cell wall metabolism was detected during the resistance response of coffee to the biotrophic fungus *H. vastatrix* (Guerra-Guimarães *et al.,* 2015).

Class III peroxidases, mainly considered as cell wall localized proteins have a relevant role in stress-induced ROS generation and consumption, in lignification, suberization, auxin catabolism, wound and defence against pathogens (Loon, 1997; Day *et al.,* 2013; Konozy *et al.,* 2013; Guerra-Guimarães *et al.,* 2016). In our work, the expression of the two *POD* genes related with ROS (*PNC2*) and lignification (*PER4*) processes was in general up-regulated at all time points of the infection process in resistant and susceptible varieties. However, significant differences were found for *PER4* at 12hpi, being more expressed in the resistant variety when

compared to the susceptible one. *PER4* gene expression continues to increase along the infection process with a maximum value at 72hpi. These results were further supported by the increase of total POD activity and by the higher staining intensity of an anionic isoform (pI 6.1) detected in the resistant variety. POD was localized in the walls or both walls and the cytoplasmic contents of infected epidermal cells, spreading later to the adjacent cortex cells. Coffee resistance related to the increase of total POD activity supported by the increase of the anionic isoforms has already been reported in pre-and post-haustorial resistance of coffee to *H. vastatrix* (Silva *et al.,* 2008). Furthermore, POD activity as a relevant element of the coffee defence response related to HR and later lignification of cell walls at the infection sites has also been stated (Silva *et al.,* 2008). Resistance associated with an increase of POD total activity has been observed in other interactions (Mohammadi & Kazemi, 2002; Takashima *et al.,* 2013; Leite *et al.,* 2014). In melon resistant cultivar to *C. lagenarium,* it was observed an increase in POD activity coincident with an intensive accumulation of ROS in infection sites and an intense cell wall reinforcement underneath the appressoria, when compared with the susceptible cultivar (Ge *et al.,* 2013). The generation of ROS with its antimicrobial role can create a toxic environment that inhibits the fungal growth, at the same time that the hydrogen peroxide can act as a signal molecule that could trigger other defence mechanisms such as HR, induction of PRs, cell wall reinforcement and lignification (Silva *et al.,* 2008; Ge *et al.,* 2013). Under the context of oxidative enzymes, polyphenol oxidase (PPO) was also study. The increase of total PPO activity was only obtained for the resistant variety at 72hpi however, this activity was not sustained by any isoform detected either in PAGE or IEF isoenzymatic profiles. According to a previous work, at 72 hours after *C. kahawae* inoculation in the resistant variety more than 50% of the infection sites exhibited hypersensitive-like cell death and therefore cell disruption (Diniz *et al.*, 2017). It has been proposed that the enhanced resistance to the pathogen was due to the increased ability of PPO to oxidize phenolic compounds upon disruption of the plastidic and vacuolar compartments by the pathogen. (Robinson *et al*., 1993; Li & Steffens, 2002; Mohammadi & Kazemi, 2002;). Furthermore, induction of PPO seems to be related with specific stimuli such as, wounding by mechanical or insect damage, and MeJA (Constabel *et al.*, 2000; Mazzafera & Robinson, 2000; Haruta *et al.*, 2001; Wang and Constabel, 2003; Melo *et al.*, 2006;). As already been referred, resistance of coffee to *C. kahawae* seems to be linked with the earlier and stronger activation of the JA pathway (Diniz *et al.*, 2017).

3.5. Conclusions

Our results showed that during the first stages of *C. kahawae* infection process (up to 24hpi) pathogen recognition, signaling and cell wall modification genes were induced in resistant and susceptible coffee varieties, probably due to a basal non-specific defence response (PTI). However, at fungal penetration stage in the resistant variety a higher expression of the pathogen recognition and signaling genes was induced together with the cell wall modification *PME41* gene. In susceptible variety, similar defence responses were also activated but later in the infection process and in an ineffective way, since the fungus was able to switch to its necrotrophic phase. The increase in POD activity was only obtained in the resistant variety and was first localized in the walls and cytoplasmic contents of epidermal cells at the infection sites, which reinforce its role in the plant defence. Furthermore, the simultaneous increase of expression of the *lignin-forming anionic peroxidase-like* gene and the POD anionic isoform activity, suggests that lignification may be induced as an effective physical barrier difficult to overcome by the pathogen, and therefore, may resulting in the halting the infection process.

To the best of our knowledge, this study provides new information into genes and proteins that may contribute to better understand key molecular and biochemical mechanisms occurring during the defence response of coffee to *C. kahawae.* The new data obtained enable to identify potential biomarkers of disease resistance that, once validated, will be useful for marker-assisted selection in coffee breeding programmes.

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Chapter 4

Final considerations

4. Final considerations

The severe socio-economic repercussions of Coffee Berry Disease, caused by the hemibiotrophic fungus *Colletotrichum kahawae*, in Africa and its potential dispersal to other coffee growing countries lead to the need of focusing research efforts on exploiting and characterising genetic resources available and using this information in breeding programmes. The coffee genotype Catimor 88, which exhibit field resistance in Kenya, was selected to characterise its resistance to *C. kahawae* (isolate Que2, from Kenya), comparatively with the susceptible variety Caturra, combining cytology, biochemistry, and RT-qPCR gene expression analysis.

The development of pre-penetration fungal growth stages was similar in the resistant and susceptible varieties. Conidia germination and appressorial differentiation were initiated at 3h and 6h post inoculation (hpi), respectively, and the majority of the appressoria were melanized at 24hpi. At these early stages of the infection, gene expression analysis revealed the up-regulation of most of the genes studied, for both coffee varieties.

Fungal penetration occurred directly by epidermal cell cuticle from the melanized appressoria, with formation of a globose infection vesicle and further intracellular hypha, by 48hpi, in both varieties, but differences in the host responses started to be more evident from this time point onwards. In fact, the hypersensitive response (HR) and the accumulation of phenolic-like compounds in cell walls and cytoplasmic content of the infected epidermal cells were observed in a significantly higher percentage of infection sites in the resistant variety, comparatively to the susceptible. In addition, 13 out of the 22 genes analysed were significantly expressed being related with the JA pathway, recognition, signaling, cell wall modifications (*PME41*), peroxidases, *PR1* and *PR10*. These genes seem to make a network of interconnected defence responses. For instance, the induction of PTL can be relate to the breakdown of cell membranes, typically associated with HR, and was coincident with the induction of the JA pathway and PR genes. Moreover, the induction of the *lignin-forming anionic peroxidase-like* gene (*PER4*) was further support by the increase in total POD activity and of an anionic isoform. POD was localized in the walls and cytoplasmic contents of epidermal cells at the infection sites, which suggest the involvement of this enzyme in the oxidation of phenolic-like compounds (associated with HR) and in the cell wall lignification (previously described as a late resistance response in this pathosystem). Furthermore, the activation of cell wall modification genes suggests that polysaccharides and arabinogalactan proteins as well as other glycoproteins can be also involved in cell wall reinforcement/strengthening during resistance.

In the susceptible variety, at fungal penetration stage (48hpi), only 3 genes (ET pathway-related and *MUR4*) were more expressed than in the resistant one. However, at 72hpi most of the genes reached their maximum expression values, particularly the ET pathways related genes, which was coincident with the beginning of the fungus necrotrophic growth suggesting the involvement of this phytohormone in host tissue senescence.

Overall, our results revealed that in both coffee varieties, the immune system enable the perception of the pathogen attack as the infection process starts. This work also highlighted the importance of the fungal penetration stage, as a key event in the activation of effective resistance responses. Based on these new data, a model of coffee - *C. kahawae* interactions is proposed (Figure 4.1).

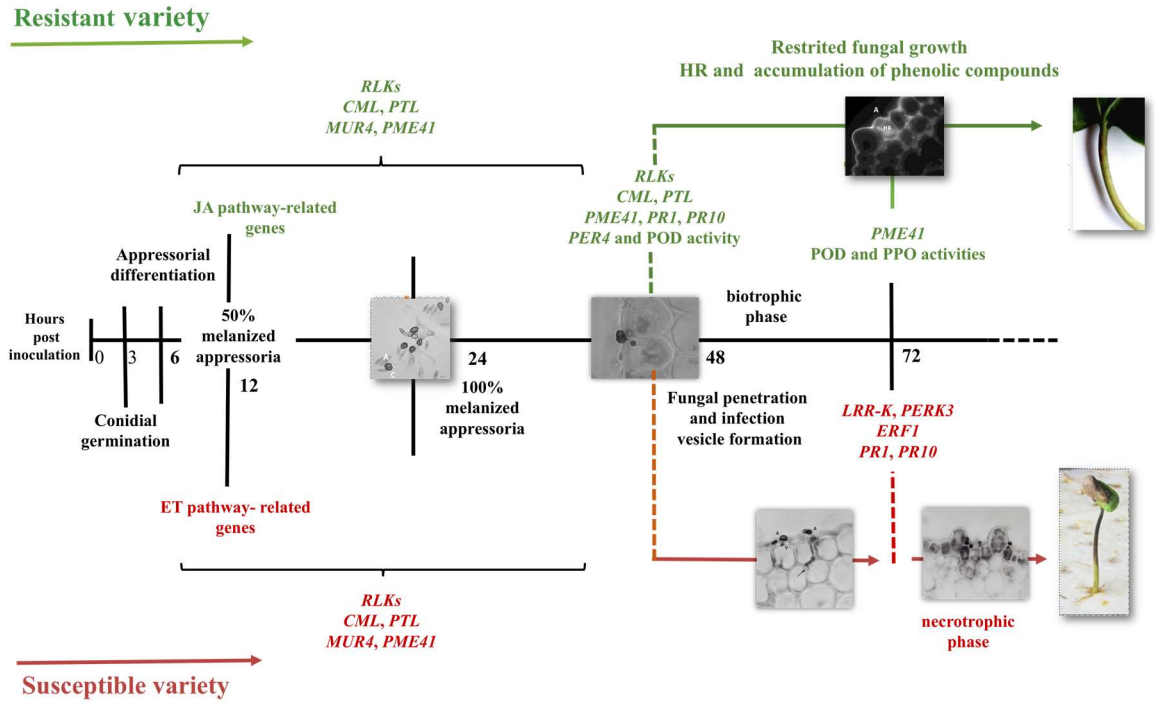

Figure 4. 1 – Proposed model for Coffee -*Colletotrichum kahawae* interactions during the time-course of the infection process. Host responses, genes and proteins referred are induced/activated in the resistant (green color) and susceptible (red color) varieties. Phytohormones: JA – jasmonic acid, ET – Ethylene. Genes: *RLK*- Receptor-like kinase, *LRR-K* - Leucine rich repeat receptor-like serine/threonine-protein kinase At2g16250, *PERK3*- Proline-rich receptor-like protein kinase, *CML* - Calmodulin-like protein, *PTL* – Patatin-like phospholipase, *MUR4* - UDP-arabinose 4-epimerase 1, *PME41* - Pectinesterase/pectinesterase inhibitor 4, *PER4* - Lignin-forming anionic peroxidase-like, *PR* – Pathogenesis-related, *ERF1* - Ethylene-responsive factor 1. Proteins: POD – peroxidase, PPO – Polyphenol oxidase.

4.1. Conclusions and perspectives

In summary, our findings provided a broader view of cellular events, candidate genes and proteins involved in coffee resistance to *C. kahawae* and are a starting point for further exploratory assays to deepen the knowledge of this pathosystem. Ultrastructural studies focused in the host responses (with emphasis on cell wall modifications) and the role of other genes and enzymes in coffee resistance and in the switch to necrotrophy are part of the ongoing work. RNA-seq from the resistant and susceptible coffee varieties at key time points of the infection with *C. kahawae* is currently ongoing to discover and identify genes related with this interaction, that have to be validated by gene expression studies.

Candidates genes and proteins putatively involved in the resistance responses of coffee to *C. kahawae*, from our data and from the RNA-seq analysis, will provide new routes for the identification of potential biomarkers of disease resistance. Once validated, those biomarkers should to be tested on other coffee genotypes with large spectra of resistance to different *C. kahawae* isolates (from CIFC collection), in order to evaluate their potential as a future useful tool for marker assisted selection.

Supplementary Material

Supplementary Material

Chapter 2

Supplementary material – chapter 2.

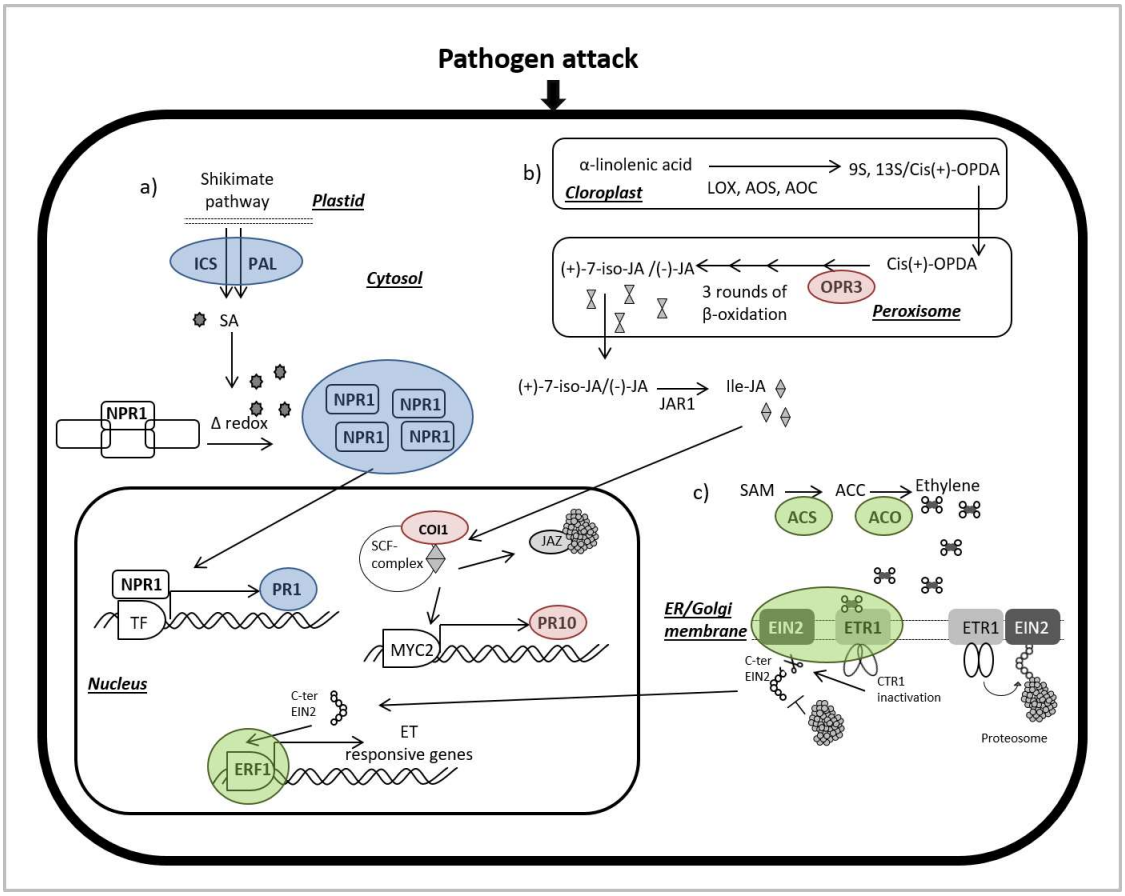

Figure S2.1 - Schematic diagram of the current models of the SA, JA and ET pathways. a) SA pathway - SA is synthesized from chorismate through two distinct enzymatic pathways: PAL-mediated phenylalanine and ICS-mediated isochlorismate (IC). SA-induced redox changes lead to the reduction of inactive NPR1 oligomers to active monomers that are translocated into the nucleus, thus activating the defence-related genes (e.g. PR1); b) JA pathway – upon release from the chloroplast membrane, α-linolenic acid is converted into OPDA by sequential steps catalysed by lipoxygenase (LOX), AOS and AOC. OPDA migrates into the peroxisome where, after reduction by OPR3 and three rounds of β-oxidation, (+)-7-iso-JA and its derivative (−)-JA is formed. By the action of JAR1 these last compounds are converted in the bioactive molecule JA-Ile. JA-dependent gene activation involves the JA-Ile binding to the receptor COI1. JAZ protein, which interacts with the SKP1-Cullin- F-box complex (SCF^{COI1}) complex, is targeted for degradation by the 26S proteasome, releasing the transcriptional factor MYC2 and promoting the expression of JA-responsive genes (e.g. PR10); c) ET pathway – ET is synthesized from SAM in a two-step reaction catalysed by ACS and ACO. In the absence of ET, the active CTR1 inactivates EIN2 and the phosphorylation of its C-terminal end is promoted resulting in suppression of the ethylene response. In the presence of ET, receptors like receptor ETR1 binds to the hormone becoming inactivated and, consequently, switching off CTR1. The C-terminal end of EIN2 is then cleaved off and migrates to the nucleus where it activates the expression of ethylene target genes, ERF1 included [adapted from Merchante *et al.* (2013) and Figueiredo *et al.* (2015)].

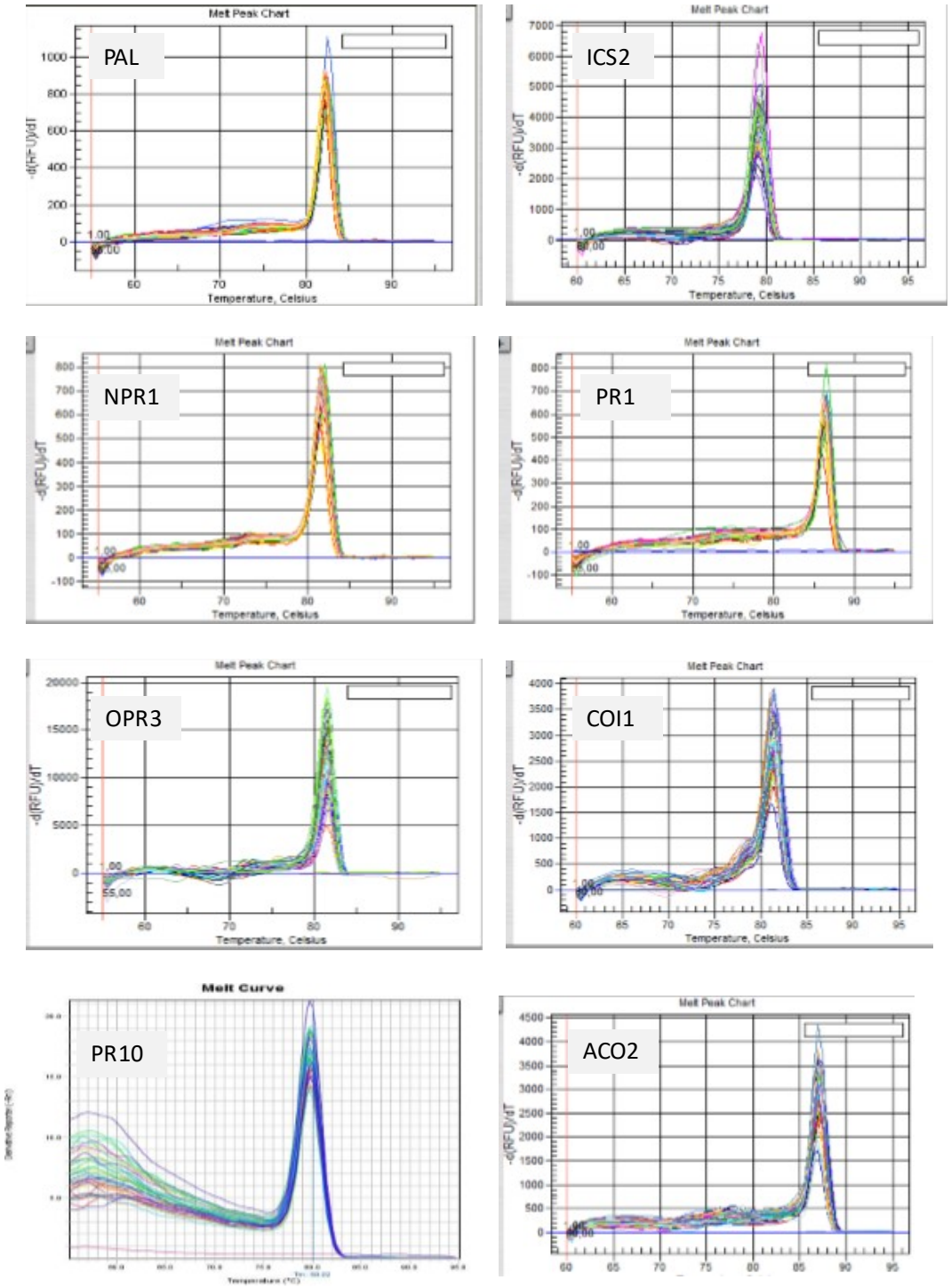

Figure S2.2 - Dissociation curves for non-specific qPCR products analysis. *PAL*, *ICS2*, *NPR1*, *PR1*, *OPR3*, *COI1*, *OPR3*, *ACO2*

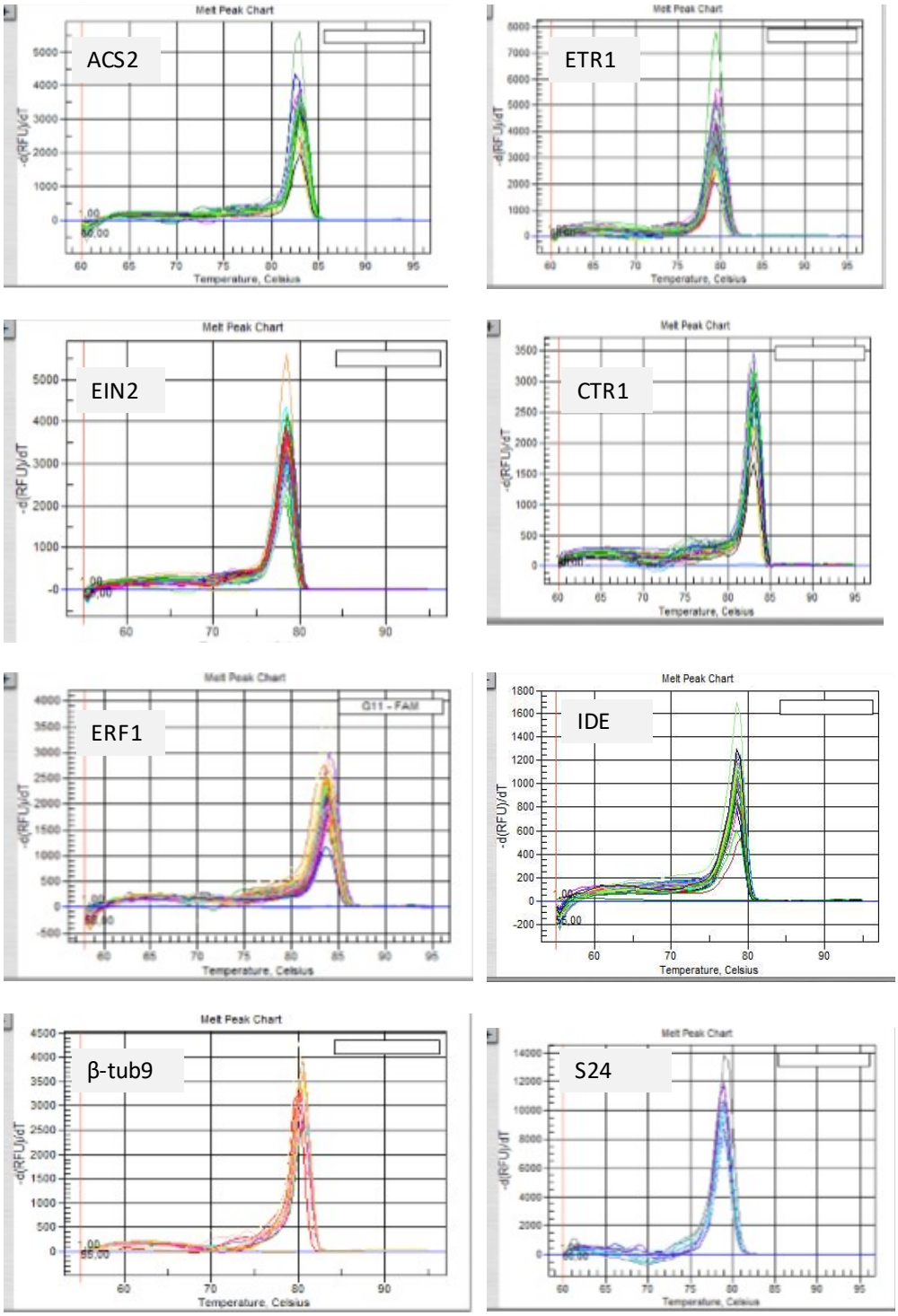

Figure S2.2 – (continued) Dissociation curves for non-specific qPCR products. *ACS5*, *ETR1*, *EIN2*, *CTR1*, *ERF1, IDE*, *β-Tub9* and S24

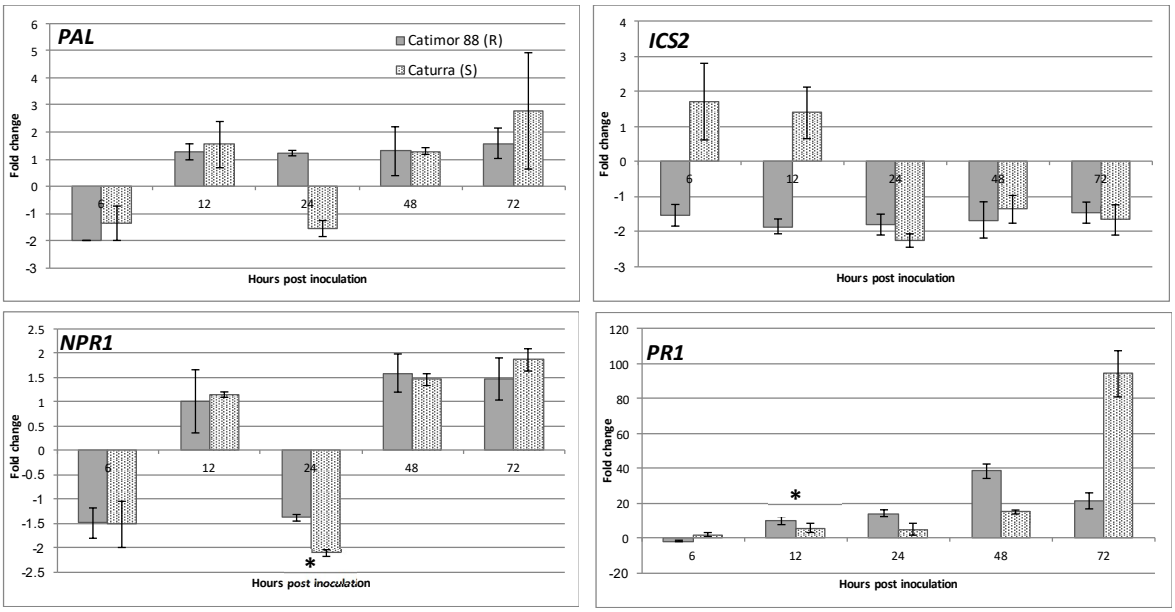

Figure S2.3 - qPCR expression analysis of SA pathway associated genes. Relative expression pattern of *PAL/ICS2* (biosynthesis), *NPR1* (receptors), and *PR1* (responsive gene) obtained in Catimor 88 (R-resistant) and Caturra (S-susceptible) coffee varieties. Mean and standard deviation of three biological replicates is presented. Fold change as relative expression of gene expression between inoculated and control samples for each of the coffee varieties/inoculation time-points. Asterisks (*) represent statistical significance ($p \leq 0.05$) of gene expression between the two coffee varieties was determined by the non-parametric Mann–Whitney U test using IBM®SPSS® Statistics version 20.0 (SPSS Inc., USA) software.

Supplementary Material

Chapter 3

Supplementary material - chapter 3

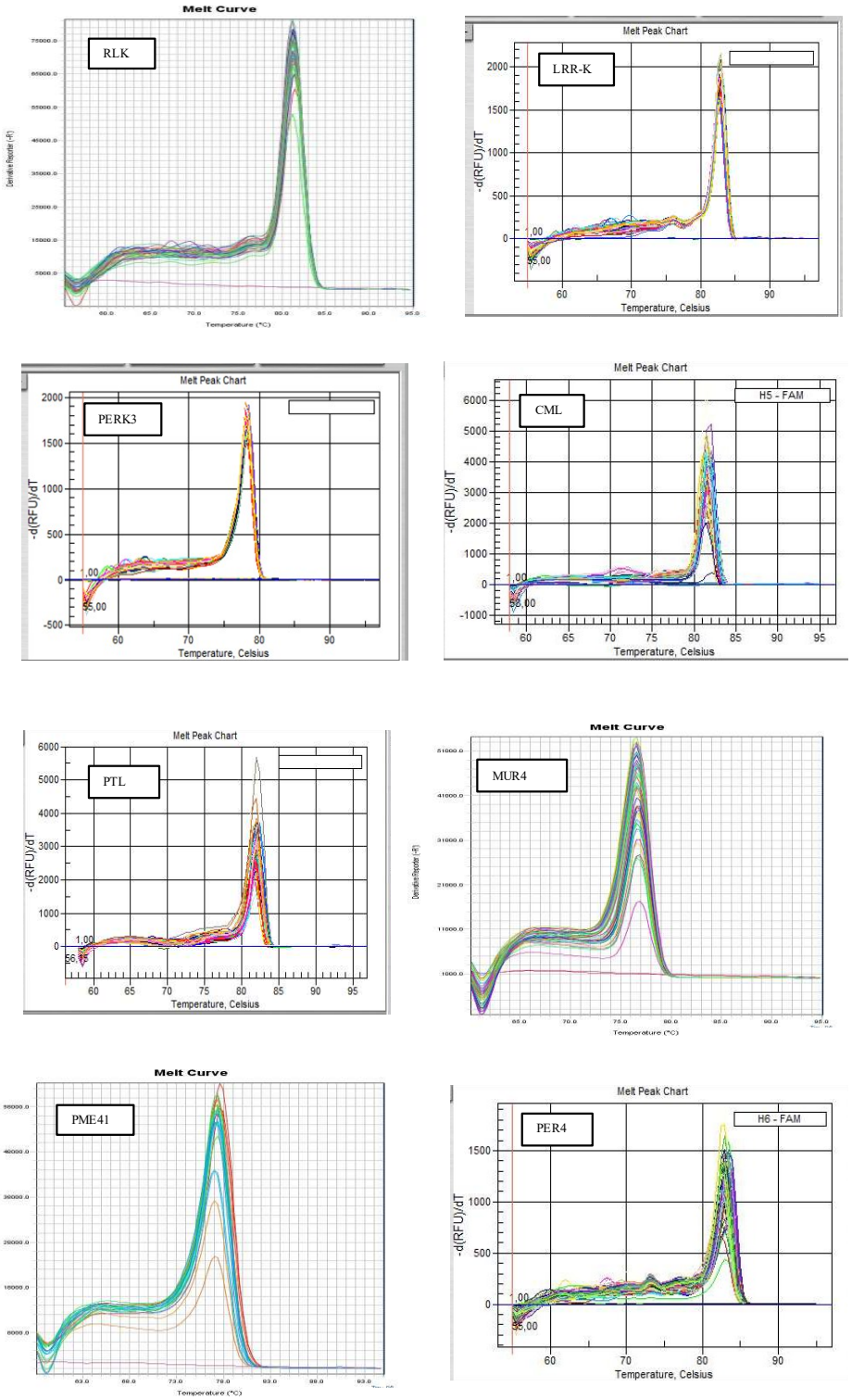

Figure S3.1 – Dissociation curves for non-specific qPCR products analysis. *RLK*, *LRR-K*, *PERK3*, *CML*, *PTL*, *MUR1*, *PME41*, *PER4*, *PNC2*, *IDE*, *β-Tub 9*, *S24*.

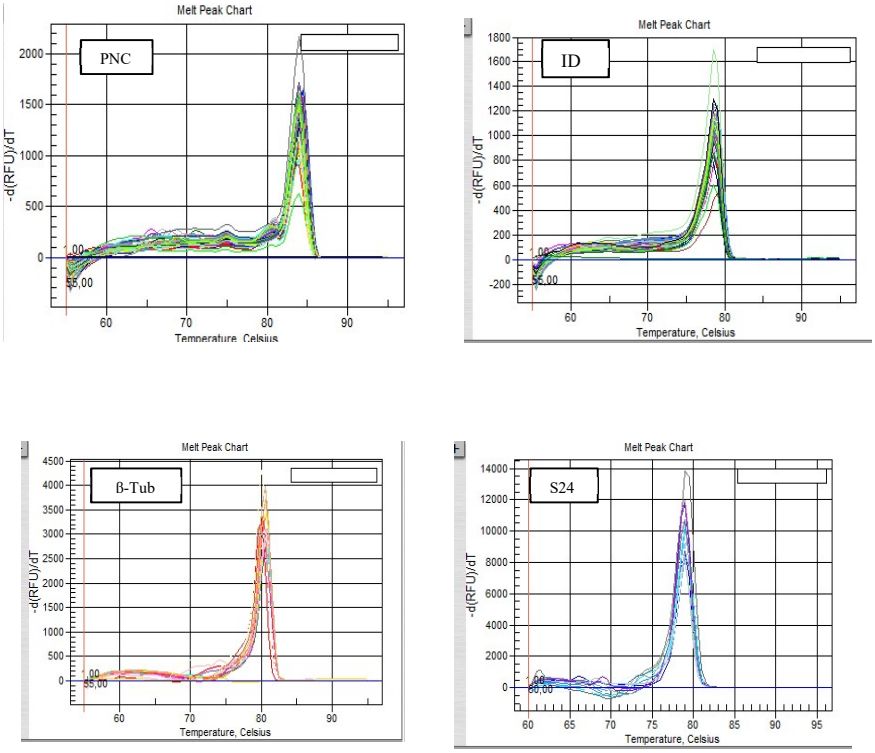

Figure S3.1 – (continued) Dissociation curves for non-specific qPCR products analysis. *RLK*, *LRR-K*, *PERK3*, *CML*, *PTL*, *MUR1*, *PME41*, *PER4*, *PNC2*, *IDE*, *β-Tub 9*, *S24*.

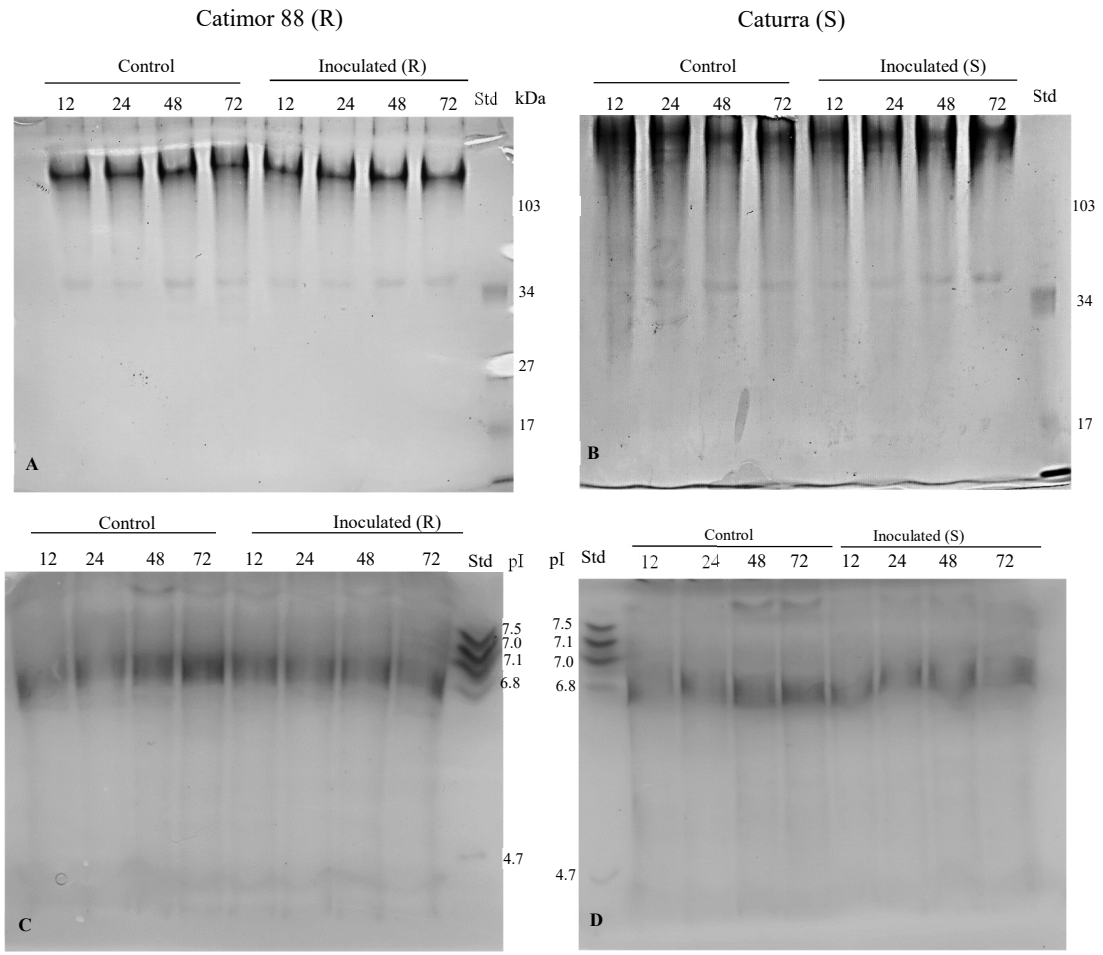

Figure S3.2 - PPO isoenzyme identification on polyacrylamide gels stained with catechol and 3-methyl-2- benzothiazolinone hydrazone hydrochloride hydrate. PAGE gels: A and B; IEF gels: C and D. PPO was extracted from control (non-inoculated) and inoculated hypocotyls of Catimor 88 (R – resistant) and Caturra (S – susceptible) varieties. Hypocotyls were collected at different times post inoculation with *Colletotrichum kahawae* (12–72 h). Twenty micrograms of protein were loaded per lane in PAGE gels and Pre-stained PAGE Low range (reference 161-0305, Bio-Rad) was used as standard (Std). Ten micrograms of protein were loaded per lane in IEF gels and IEF Standard (reference 161-0310, Bio-rad) was used as standard (Std); pI – Isoelectric point.